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In Vitro Cytotoxicity Assessment

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Summary

The most frequent reason cited for withdrawal of an approved drug is toxicity, yet no simple solution exists to adequately predict such adverse effects. Compound prioritization and optimization during in vitro screening cascades need to be based on confidence, not only in efficacy and bioavailability, but also in safety. A wider number and diversity of potential molecular and cellular effects of compound interactions might affect safety than might affect efficacy or bioavailability. Accordingly, cytotoxicity assessment is less specific, more multiparametric, and extrapolatable with less certainty, unless there are specific safety signals indicated by the chemical structure or by precedents. Cytotoxicity assessments have been limited by their inability to measure multiple, mechanistic parameters that capture a wide spectrum of potential cytopathological changes. Assays with multiple parameters for key, multiple, and different features, such as in high content screening (HCS), are more predictive because they cover a wider spectrum of effects. Assays need to be applied to a large set of marketed drugs that produce toxicity by numerous and different mechanisms for assessment of correlation with human toxicity. This will enable determination of the concordance between in vitro and in vivo results. Multiparametric, live cell, prelethal cytotoxic HCS assays for assessing the potential of compounds for causing human toxicity address some of the limitations of traditional in vitro methods. Assays of this class were used to screen a library of drugs with varying degrees of toxicity and it was found that the sensitivity of the assays was 87%, whereas assay specificity was more than 90%, thereby minimizing false positives.

Key Words: Autophagy; drug discovery; energy homeostasis; genotoxicity; hepatotoxicity; immune-mediation; oxidative stress.

1. Introduction

Early in drug discovery, in vitro cytotoxicity is becoming increasingly recognized as an effective indicator of human toxicity potential that must be addressed in order to maximize probability of successful progression of compounds into development *(1–6)*. Compound prioritization and optimization during in vitro screening cascades need to be based on confidence, not only in efficacy and bioavailability, but also in safety *(1)*. However, safety is more multifactorial, as it is dependent on homeostasis of virtually all cellular processes *(7)*. A wider number and diversity of potential molecular and cellular effects of compound interactions might affect safety than might affect efficacy or bioavailability. Accordingly, cytotoxicity assessment is less specific, more multiparametric, and extrapolatable with less certainty, unless there are specific safety signals indicated by the chemical structure or by precedents. Extrapolation needs a greater foundation of mechanistic understanding of both in vitro and in vivo pathogenesis of toxicities, as well as rigorous, empirical validation of models. Finally, it is important to recognize that cytotoxicity models are

From: *Methods in Molecular Biology, vol. 356:*
High Content Screening: A Powerful Approach to Systems Cell Biology and Drug Discovery
Edited by: D. L. Taylor, J. R. Haskins, and K. Giuliano © Humana Press, Inc., Totowa, NJ

inevitably limited by their inability to account for toxicities arising from interactions between different cell types and structures and extracellular matrices, such as occur at the tissue, organ, and system level *(1)*. The previously mentioned complexity indicates that thorough and systematic consideration and evaluation of in vitro models should be undertaken before their implementation.

2. Mechanisms of Cytotoxicity

2.1. Nuclear Effects and Cell Proliferation

Cell proliferation is dependent on intact structure and function of all vital cell processes. Consequently it is affected early in all toxicities, even if only secondarily. Additionally, one of the numerous specific processes involved in replication might be affected primarily as the target of compound effect. Proliferation provides a "catch-all" screen for cytotoxic effects *(8,9)*. Characteristic morphological changes have long been noted to occur early with cell death, including nuclear condensation, shrinkage, and fragmentation. The opposite effect might occur with cell cycle inhibitors that cause nuclear swelling and nuclear-cytoplasmic asynchrony. Toxicity that is DNA-based and inheritable, that is genotoxicity, and occurs owing to mutagenicity or disruption of chromosomal separation during cell division is discussed at length elsewhere.

2.2. Cell Membrane Effects and Transport

The most well-known and assayed cytotoxic effects are cell membrane disruption causing leakage of cellular contents into the extracellular space or influx of extracellular dyes that stain cellular constituents *(1,6,8,9)*. These effects are typically nonspecific and occur when energy and ion homeostasis are compromised to the point in which the membrane barrier can no longer be maintained. However, they might occur more owing to specific interactions such as with membrane perturbing agents like detergents and volatile anesthetics, inhibitors of vital functions like ion transport or signal transduction, and with inhibitors of specific transporters of noxious substances such as in biliary and renal tubular cells. Cell membrane disruption might occur without necrosis if the effect is mild. For example, hepatic accumulation of lipid and carbohydrate in glycogenosis and steatosis might result in release of cellular enzymes without any evidence of necrosis. Release occurs by blebbing, in which cytoskeleton is disrupted and plasma membrane evaginates to form vesicles that bud off without loss of cell membrane continuity. Blebs contain cytoplasm complete with enzyme and even organelles. Alternatively, cellular constituents might be released by cell rupture, with more severe injury. Loss of the cell membrane's barrier function is frequently used in viability assessments to measure cellular influx of stains, such as Trypan blue, or DNA stains. Such stains might grade severity of injury on the basis of size and permeation properties of the dye.

2.3. Mitochondrial Effects and Energy Homeostasis

The third most well-studied and recognized mechanism of cytotoxicity is probably mitochondrial toxicity *(10–12)*. Mitochondria are ubiquitously involved secondarily to virtually all other cellular effects. Also, they are frequently a primary target of toxicities because of their complexity and numerous critical and diverse roles in energy and calcium homeostasis, biosynthesis, oxidative stress, and apoptosis. **Table 1** identifies and provides examples of the major mitochondrial functions that might be inhibited by drugs. Most of these mitochondrial dysfunctions are manifested as alterations in mitochondrial membrane potential or in reductive activity of the enzymatic oxidoreductases. Frequently, if cells are not overwhelmed by the toxic effects they make early compensatory adaptations, such as increasing mitochondrial biogenesis *(11,13)*.

2.4. Reactive Metabolites, Oxidative Stress, and Immune-Mediation

Many drugs have long been recognized to produce toxicity from oxidative stress by several mechanisms: (1) progressive reduction of oxygen into reactive oxygen species such as superoxide, peroxides, and hydroxyl radical and reactive nitrogen species such as nitric oxide and peroxynitrite

Table 1
Mechanisms of Mitochondrial Toxicity

1. *Oxidative phosphorylation*
   - Inhibition of complexes, for example, I by rotenone and fenofibrate, II by, IV by cyanide, V by oligomycin; depleters of coenzyme Q such as amitryptyline
   - Redox cyclers diverting electrons to form reactive oxygen and nitrogen species, for example, quinones
   - Uncouplers of electron transport from ATP synthesis, for example, protonophores, tolcapone, flutamide, cocaine, furosemide, fatty acids
2. *Fatty acid β-oxidation*
   - Inhibition by valproate, tetracyclines, nonsteroidal antiinflammatory drugs, antianginal cationic amphiphilic drugs, female sex hormones, CoA depleters such as valproate and salicylate
3. *Krebs cycle*
   - Inhibition of aconitase by superoxide and fluoroacetate, of succinate dehydrogenase by methamphetamine and malonate, of α-ketoglutarate dehydrogenase by salicylic acid
4. *Mitochondrial membrane transporters*
   - Inhibition of adenine nucleotide transporter by zidovudine
5. *Mitochondrial permeability transition pore*
   - Opening by reactive oxygen species, reactive nitrogen species, bile acids, thio crosslinkers, atractyloside, betuliniate, lonidamidem various anticancer drugs, to collapse mitochondrial membrane potential and activate mitochondrial apoptotic pathway
6. *Mitochondrial proliferation*
   - Inhibitor of DNA polymerase-γ for mitochondrial DNA synthesis by nucleoside reverse transcriptase inhibitors
   - Inhibition of mitochondrial protein synthesis by oxazolidinone antibiotics
   - Mitochondrial DNA mutation, for example, by oxidative injury by ethanol
7. *Mitochondrial oxidative stress*
   - Glutathione depletion by acetaminophen, bromobenzene, chloroform, allyl alcohol
   - Redox cyclers (*see* oxidative phosphorylation)
   - Reactive metabolites

(*see* **Fig. 1**); (2) depleting glutathione, (3) bioactivation to an electrophilic metabolite that forms adducts with cellular macromolecules ***(14–17)***. As oxidative stress is a ubiquitous process found with normal intermediary metabolic activity and xenobiotic detoxification, cells have evolved complex antioxidant defensive systems that are readily upregulated, including signal transduction by transcription factor translocation (ap-1, nrf2), and numerous protective enzymes and free radical scavengers (*see* **Fig. 1**). Reactive metabolite formation has been associated with immune-mediated mechanisms of toxicity affecting skin, blood cells, and liver. The mechanism has not been defined but might relate to hapten formation and cell stress, which in the presence of an activate immune system and inflammation trigger an autoimmune response ***(14)***.

2.5. Lysosomal Effects and Autophagy

One of the most commonly recognized toxicities that is specifically linked to chemical structure and primarily involves lysosomes is phospholipidosis ***(1)***. This occurs because of interaction of cationic amphiphilic drugs with phospholipases or phospholipids such that their lysosomal catabolism is inhibited and they subsequently accumulate to excessive extent. Phospholipidotic drugs have a hydrophobic ring with hydrophilic side chain containing a charged cationic amino group and include amiodarone, perhexiline, chloroquine, clomipramine, imipramine, fluoxetine, norfluoxetine, gentamycin, propranolol, tamoxifen, and quinacrine. There are potential serious toxic effects of chronic administration. For example, amiodarone is an antiarrhythmic that produces serious pulmonary toxicity in 10% patients under chronic treatment and also liver injury.

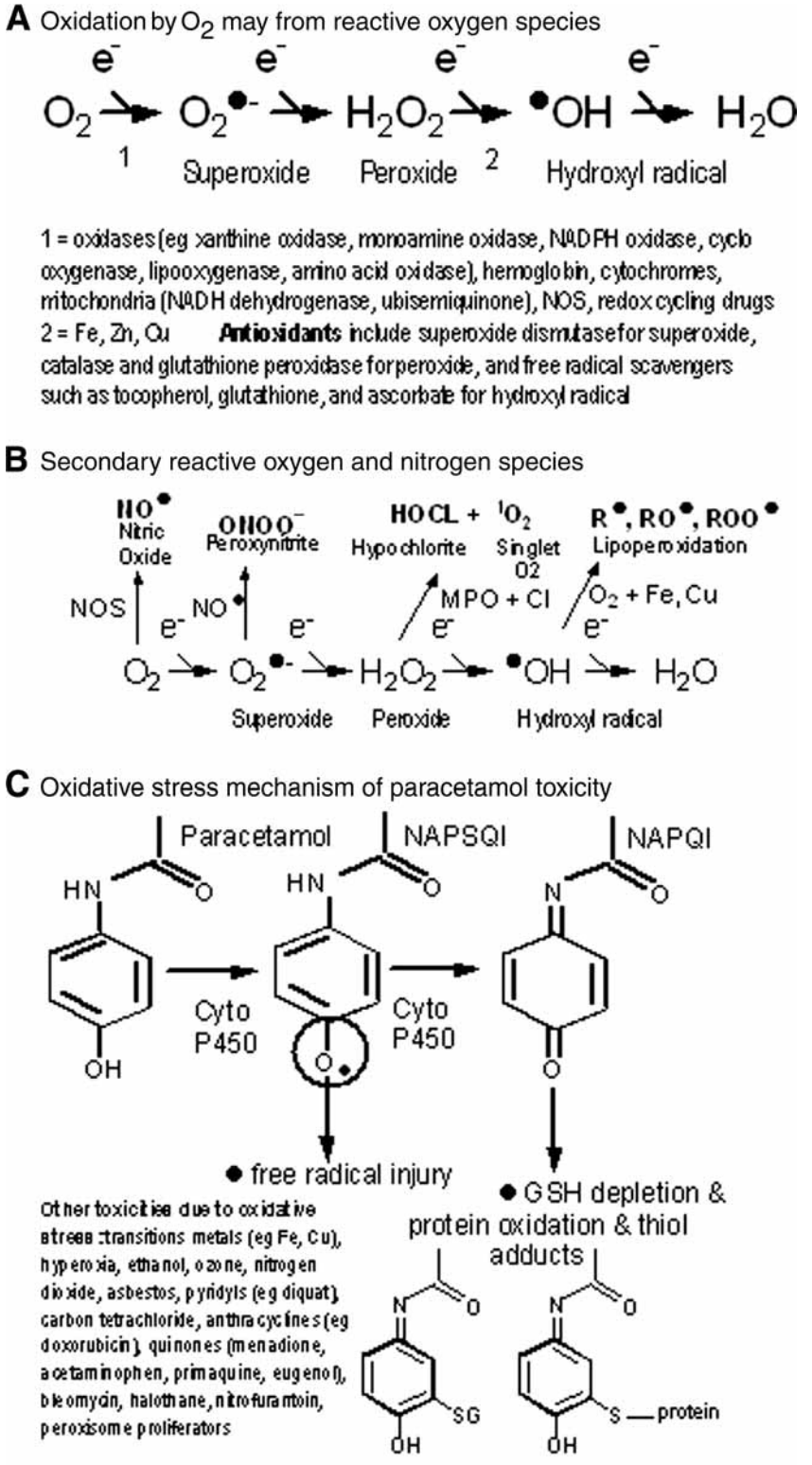

Fig. 1. Mechanisms of oxidative stress.

Perhexiline used for ischemic heart disease might produce severe liver injury in 1–2% of patients under chronic treatment.

A second and less commonly recognized primary lysosomal effect that is related drug structure has been referred to as vacuolization ***(18,19)***. This also occurs because of lysosomal swelling, especially with tertiary amines that are charge neutral at physiological pH. In vitro it occurs at concentrations from 0.1 to 2.5 m*M*. Organic bases enter the acidic lysosome and are trapped by protonation at the lower pH that makes them positively charged, and prevent back diffusion. The drugs accumulate and increase intravacuolar osmotic activity. Consequently, there is water influx and swelling into large, clear vacuoles.

Lysosomes are also involved secondarily affected with other cytotoxicities. Their activity and mass might increase with apoptosis in which cells are undergoing programmed cell death, including autophagy as the cells involute. Their size and mass might decrease with general ill health of the cell.

2.6. Calcium Regulatory System

Calcium plays an integral role not only in regulating cellular functions such as secretion, contraction, metabolism, and gene transcription, but also in apoptosis and cell death. There is a 10,000-fold gradient of ionized calcium from the extracellular to intracellular space, that if not maintained and controlled, leads to dysfunction, activation of degenerative enzymes, and cell loss. Accordingly, calcium is an important prelethal signal of cell injury of all causes ***(8,20,21)***. Calcium dyshomeostasis might also result from direct interaction of drugs or their toxic metabolites with Ca-regulatory channels and pumps, such as with fibrates, statins, anthraquinones such as doxorubicin, and thapsigargin.

3. Limitations of Traditional in Vitro Methods

Several limitations of traditional in vitro methods of assessment of cytotoxicity have resulted in their use being substantially restricted (*see* **Table 2**). Probably the most ineffective assays are those that have not allowed sufficient duration of exposure for cytotoxicity to be expressed ***(6,8)***. An estimated three-quarters of drugs do not express their toxicity with acute exposure, but only after several days exposure. Some drugs, such as mitochondrial DNA and protein synthesis inhibitors might take up to a week to show cytotoxic effects in many assays. Dose–response curves are typically shifted to the left, toward increased sensitivity, with increasing duration of exposure from hours to days.

Another common cause of failure of some assays to detect in vivo toxicity potential is their dependence on measurement of events that occur only late in the life of the cell when severe cell injury has occurred, such as cell rupture with release of cellular constituents and uptake of extracellular substances. Such assays lack sensitivity in predicting in vivo toxicity primarily because cell death is not required for significant toxicity. Also, limited solubility of drugs might limit testing the drug to a sufficiently high concentration. Assay of total ATP content of cell populations is frequently used for cytotoxicity assessment, but has limited utility, especially when used alone as an indicator, because it changes quite late in pathogenesis ***(22,23)***. The lateness of this change reflects the tight regulation and buffering of ATP concentration by interconversions with other high-energy phosphates, such as creatine phosphate, and by replenishment by metabolic conversion of substrate stores, such as glycogen. This tight regulation is required because ATP in turn regulates activities of numerous metabolic pathways, cell functions, and cell structure. Current assays for ATP are also significantly limited because of the need to apply them to cell populations rather than single cells (*see* below).

Cytotoxicity measurement of events that are too early in the pathogenesis of cytotoxicity might also be ineffective for assessment of in vivo toxicity potential. For example, measurement of concentration of a noxious substance, byproduct or metabolite, or of activation of a signal

Table 2
Major Criteria for an Effective Cytotoxicity Assay

1. *Prelethal: not too late or too early an indicator of adverse effect*
   - Prelethal effect as opposed to measurement of cell death is required
   - Measurement should not be made of noxious substance or a signal transduction event as opposed to measurement of adaptive and adverse effect
2. *Chronic: allows significant duration of exposure for toxicity expression*
   - Most toxicities are expressed only after multiple days of exposure
3. *Catches all: includes measurements of adverse effect in common to all toxicities*
   - There needs to be a "catch-all" measure of an activity that is affected in a final common pathway of cell injury
4. *Mulitplexing: makes multiple measurements of different end points for different processes*
   - A single end-point assay will not identify most cytotoxicities
5. *Measures morphological and functional characteristics*
   - Structural and functional measurement is complementary and additive
6. *Mechanistic: provides mechanistic information*
   - Signals generated in cytotoxicity assays need to indicate mechanisms to be followed up to derisk the compound
7. *Tracks individual cells*
   - Allows identification of hormesis and separation of compensatory adaptation from degenerative change
   - Allows more accurate identification of sequence of change in different cytotoxicity parameters as cells might not be synchronous or alike in their response
8. *Uses live cells under normophysiological conditions:* cell function is substantially affected by temperature, humidity, and oxygenation, pH and osmolality, as well as media growth factors and attachment substrate
9. *Predictive: high sensitivity and specificity and concordance with in vivo toxicity in species designed to be indicative of*
   - Inhibitor of DNA polymerize gamma for mitochondrial DNA synthesis by nucleoside reverse transcriptase inhibitors
10. *Precise: high level of within-run, across-day, and across-laboratory precision*
    - Assay must be reproducible
11. *Practical: sufficient operational performance: throughput and cost-effectiveness*
    - At least 100 to several hundred per week
    - Assays are available that can be run at significantly less than 100$ per compound including materials, staff, and instrumentation
12. *Dose–responsive: determines different response over range of concentrations*
13. *Effective cell model:*
    - Drug-metabolism competent
    - Same species as predicting for

transduction pathway is insufficient to conclude that a compound is toxic. Direct demonstration and measurement of the adverse effect, or of an adaptation to it, are required.

Cytotoxicity assessments have been limited by their inability to measure multiple, mechanistic parameters that capture a wide spectrum of potential cytopathological changes. The complexity of the biology behind a toxicological change requires several criteria for an effective cytotoxicity assay. First, there is no single parameter likely to be able to identify all toxicities, which immediately limits utility of uniparametric assays. Assays with multiple parameters for key, multiple and different features are more predictive because they cover a wider spectrum of effects. Additionally, those assay parameters that represent fundamental, cellular mechanisms of pathogenesis rather than being purely descriptive are more potent. Assays that combine morphology and functional assessments are more predictive as they measure more parameters

by using more and independent analytic approaches, such as dimensional image analysis and fluorescence intensity measurements. Morphological assessments provide information about size and shape of cells and organelles, as well as on intracellular location, such as with transcription factor translocation or lysosomal sequestration. Thus assays measuring both morphology and function are making a more comprehensive evaluation over a wider spectrum of change.

Assays that measure end points for cell populations rather than multiple individual cells might produce contradictory findings ***(6)***. For example, mitochondrial reductive capacity will be decreased with decreased cell numbers but increased with cells that have been activated, such as lymphocytic immune-activation, or if the cells have adapted to the stress associated with the toxicity, such as with mitochondrial biogenesis. Thus, mitochondrial reductive capacity might be either increased or decreased with toxicity. Similar contradictory interpretations might occur with other cellular activities, for which there is a compensatory adaptive increase before their failure. This biphasic change is referred to as hormesis and occurs with not only reductive mitochondrial activity, but with mitochondrial number, cell number, mitochondrial membrane potential, antioxidant system activity, and numerous other activities. Finally, individual cell studies might be more accurate than cell population studies in which responses are variable over time or over different cells. Analysis of the sequence of changes in the different parameters might be important in elucidation of mechanisms and pathogenesis of toxicity.

One of the largest limitations of in vitro assays is their lack of full validation and determination of their sensitivity and specificity for prediction of human toxicity potential. Assays need to be applied to a large set of marketed drugs that produce toxicity by numerous and different mechanisms for assessment of correlation with human toxicity. This will enable determination of the concordance between in vitro and in vivo results. Typically, such assays show high specificity, in excess of 90% ***(6)***. When compounds react positively in the cytotoxicity evaluation, this is associated with in vivo toxicity. The major concern though, is the sensitivity with which the toxic potential is assessed. In a comparison of seven conventional cytotoxicity assays applied to 600 compounds with single end points measurement in an acute exposure experiment, only glutathione had significant sensitivity, 19% ***(6)***. Measures of mitochondrial reductive capacity and DNA synthesis were half as sensitive. Caspase induction, synthesis, protein synthesis, superoxide production, and membrane integrity were of negligible value.

4. Cytotoxicity Assessment in Drug Discovery

4.1. Screening for and Derisking Human Toxicity Potential

Acute toxicity is frequently assessed in drug discovery programs, in which cell-based efficacy assays are used and in which cytotoxicity could be a false-positive for efficacy. Although, efficacy is frequently expressed by cells in short-term studies of hours duration, toxicity is most often expressed only over a duration of several days. Furthermore, concentrations causing toxicity are frequently greater than those for efficacy, and so efficacy assays are limited for this reason alone in detecting in vivo toxicity potential. As discussed above, acute toxicity is typically effective at identifying in vivo toxicity potential only for overtly and potently cytotoxic compounds (**Table 3**).

Go or no go decision-making might frequently be made based on occurrence of marked and overt cytotoxicity at concentrations near the estimated efficacy concentration. However, chronic rather than acute cytotoxicity assessment is needed for prediction of human toxicity potential for most effective optimization and prioritization of compounds based on confidence in safety. Such early ranking of compounds for their progression is important for early initiation of potential hazard identification and for flagging up compounds needing follow-up safety assessment, and early development of risk management strategies.

Table 3
Cellular Functions Assessed in Cytotoxicity Assays

1. *Cell membrane and permeability barrier to extracellular environment*
   - Leakage of cell contents, for example, lactate dehydrogenase, chromium release
   - Entry of extracellular dyes, for example, Trypan blue, DNA stains
2. *Nucleus and cell proliferation*
   - Cell number
   - Nuclear size: contraction with apoptosis and swelling with cell cycle inhibition
   - Frequency distribution of nuclear DNA content of cell population
   - Protein synthesis, for example, ^{14}C-labelled methionine incorporation
   - DNA synthesis, for example, tritiated thymidine incorporation, DNA stains
   - Mass tracker dyes, for example, for lysosomes or mitochondria
3. *Mitochondria and energy homeostasis*
   - Dye oxidation, for example, tetrazolium salts (e.g., MTT, MTS), alamar blue
   - ATP concentration, for example, luciferase assay
   - Oxygen consumption, for example, oxygen electrodes, Luxcell oxygen sensor
   - Mitochondrial protein and nucleic acid synthesis
   - Mitochondrial mass, for example, mitotracker dyes
4. *Antioxidant system and oxidative stress management*
   - Oxidant production, for example, dihydroethidium, dichlorofluorescein
   - Antioxidant changes, for example, glutathione, antioxidant system enzyme, resistance to dye oxidation (e.g., total antioxidant status)
   - Macromolecular oxidation byproducts, for example, malondialdehyde, hydroxynonenal, 8-hydroxyguanosine
5. *Lysosomes and macromolecular disposal*
   - Phospholipidosis—Nile red, lysotracker dyes, electron microscopy of lysosomal multilamellar bodies
   - Vacuolization
   - Autophagy
6. *Cytoskeleton and cell shape*
   - Blebbing
7. *Ca regulatory system and Ca homeostasis*
   - Ionized and total calcium concentration
8. *Signal transduction and adaptive gene expression*
   - Cytoplasmic-nuclear translocation, for example, proportioning of immunocytochemical stain for NFKB with inflammation and AP-1 and NRF2 with oxidative stress
9. *Cell involution and apoptosis*
   - Nuclear condensation and lobulation, caspase activation, phosphatidyl serine externalization, annexin, immunocytochemistry, DNA fragmentation and labeled-dUTP incorporation by terminal deoxynucleotidyl transferase
10. *Intermediary metabolism and specific enzyme activities or metabolite concentrations*
    - Bench-top or automated chemistry analyzer assays of cell lysates for key enzymes of intermediary metabolism, antioxidant system, ion transport
11. *Cell-based efficacy targets in drug discovery*
    - Live-cell assays in which assessment of efficacy is based on a cellular function that might be inhibited by cytotoxicity

4.2. Strategies for Implementation of Cytotoxicity Assessment in Discovery

Cytotoxicity data cannot be used in isolation for decision making, excepting in which there are profound effects at concentrations similar to those for efficacy. Toxicity potential needs to be considered in the context of efficacy and bioavailability properties. Typically evaluation of cytotoxicity needs to be integrated with off-target pharmacological activity, drug metabolism and

transport and distribution, chemical properties, in vivo animal study results. It is also important to consider differences in protein binding in vitro compared to in vivo, whether toxicity is mediated by a toxic metabolite, in which the drug is concentrated in vivo, relationship of in vivo exposure of Cmax needed for efficacy vs exposure or concentration causing toxicity in vitro.

A second tier of cytotoxicity testing based on the type of toxicity identified from the first, screening tier is usually warranted if the compound or series of compound progresses. This approach enables progressively more specific in vitro studies to define narrower dose–response relationships around the cytotoxic concentration, mechanisms of toxicity, and biomarkers for risk management.

4.3. Basic Assay Design

Optimal features for cytotoxicity assay design are outlined in **Table 2** and are based on considerations previously made. For adequate sensitivity, there must be multiple parameters incorporated into the assay and these must represent different relevant processes that occur early in cell injury, rather than before or after. Measurements must be precise and accurate. There must be sufficient duration of exposure for the toxicity to be expressed at relevant concentrations. For drugs that exert their toxicity through reactive metabolites, the cell model must have some metabolic competence for the relevant species. For assessment of the relevance of the cytotoxicity and designing follow-up studies, parameters must provide some mechanistic understanding. For practicality, assays must be cost-effective, have moderate throughput and be widely accessible for implementation.

5. Methods for In Vitro Assessment of General Cytotoxicity (Necrosis)

5.1. Conventional Methods

Conventional methods used for cytotoxicity assessment are typically adapted for use in multiwell plates for fluorescent plate readers and flow cytometers in order for relevant throughput to be available (**Table 3**). Measures are typically based on fluorescence intensity from dyes that directly interact with intracellular constituents and reflect their concentration or activity, or from fluorescent labels attached to antibodies specific for cellular antigens. The latter immunocytochemical stains and the dyes for measurement of parameters that are static over the duration of the measurement, can be applied to dead or fixed cells or, if morphological information is not obtained, to their lysates. Such dyes would include those for DNA or lipid, cell or organelle mass, or ATP. However, measurement of dynamically changing parameters such as free ion concentrations, reactive oxygen species, or membrane potential need to be measured in live cells. Such measurements require continuous maintenance of normophysiological conditions of temperature, humidity, and oxygen tension.

Alternatives to fluorescence-based staining or using specific fluorescence probes have been used for cytotoxicity screening. Using reverse transcriptase polymerase chain reaction, small profiles of specific gene expression patterns might be applied effectively *(24)*. Assays for specific enzymes or metabolites of intermediary metabolism and oxidative stress have been adapted to automated chemistry analyzers and applied to cell-based systems for assessment of toxicity *(8)*.

There is a wide array of fluorescent probes currently available including for nuclear and mitochondrial DNA, lysosomal and mitochondrial mass. Immunocytochemistry can be used for morphological assessments, intracellular localization, quantitation of specific antigens, immunophenotyping, and transcription factor translocation.

5.2. High Content Methods

The recent development of capacity of fluorescence plate readers and, more recently, flow cytometers for multiple fluorescence measurements combined with image analysis has markedly enhanced their value in cytotoxicity assessment. These new capacities can now be used to simultaneously measure multiple parameters for both morphological and biochemical cell features *(25–27)*. Furthermore, they can be applied at the single cell level, as well as at the

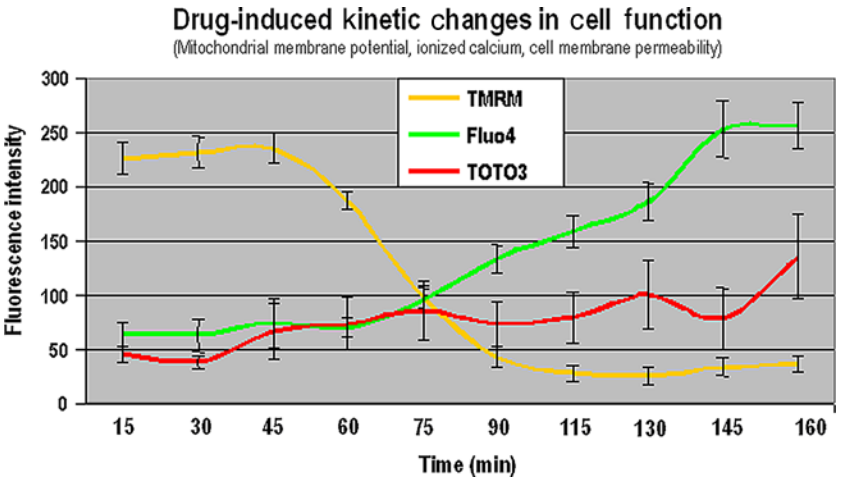

Fig. 2. Multiparametric, live cell, prelethal cytotoxicity assay.

well level and level of cell populations or subpopulations derived from thresholding for certain values of a specific parameter. Finally, they are able to identify intracellular location.

Separable fluorescence signals can be measured simultaneously for different cellular parameters by choice of dyes with nonoverlapping spectral properties. Dyes with overlapping fluorescence might also be used to discriminate different cellular targets simultaneously if they have nonoverlapping subcellular locations, such as nuclear and mitochondrial DNA. Also, dye replacement could allow use of dyes with similar fluorescence, such as application of an immunocytochemical stain following loss of a vital stain with cell death or fixation.

6. Assay Example: Quad-Probe Assay for Predictive Hepatotoxicity

A multiparametric, live cell, prelethal cytotoxicity assay for assessing the potential of compounds for causing human toxicity has been recently reported using high content screening *(1,6*; *see* **Fig. 2***)*. This was based on the "quad probe" assay reported by Haskins and colleagues *(25–27)*. Human hepatocellular carcinoma cultured in 96-well plates and loaded with four fluorescent dyes, fluo-4 for intracellular calcium, tetramethylrhodamine methyl ester for mitochondrial transmembrane potential, Hoechst 33342 for DNA content to determine nuclear area and cell number, and TOTO-3 for plasma membrane permeability. In order to increase the assay sensitivity, the cells were continuously exposed to drugs for 3 d at a range of concentrations, up to at least 30 times the plasma concentration needed for an efficacious effect. Assay results were compared with those from seven conventional, in vitro cytotoxicity assays that were applied to 600 drugs and compounds and shown to have low sensitivity (<25%), although high specificity (approx 90%) for detection of toxic drugs. For 250 drugs with varying degrees of toxicity, the sensitivity of the novel multiparametric in vitro assays was 90%. Specificity of these in vitro assays was more than 95%, thereby minimizing false-positive results.

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