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Teaser High-content analysis is being adapted to examine nanoparticle trafficking in cells and to assess the sublethal mechanistic effects of polymers, excipients, and permeation enhancers.

High-content analysis for drug delivery and nanoparticle applications

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High-content analysis (HCA) provides quantitative multiparametric cellular fluorescence data. From its origins in discovery toxicology, it is now addressing fundamental questions in drug delivery. Nanoparticles (NPs), polymers, and intestinal permeation enhancers are being harnessed in drug delivery systems to modulate plasma membrane properties and the intracellular environment. Identifying comparative mechanistic cytotoxicity on sublethal events is crucial to expedite the development of such systems. NP uptake and intracellular routing pathways are also being dissected using chemical and genetic perturbations, with the potential to assess the intracellular fate of targeted and untargeted particles *in vitro*. As we discuss here, HCA is set to make a major impact in preclinical delivery research by elucidating the intracellular pathways of NPs and the *in vitro* mechanistic-based toxicology of formulation constituents.

A brief recap of cytotoxicity assays

Q4 *In vitro* cell-based assays have long been used to assess the cytotoxic effects of drug exposure [1]. Cells undergoing acute necrosis swell and lose metabolic capacity, thereby losing the capacity to maintain a barrier to the extracellular space and the ability to reproduce. Initial assays measured cell counts and morphological changes associated with cell death. Effects were detected by assessment of the maintenance of plasma membrane integrity, as reflected by exclusion of dyes, including trypan blue, eosin, propidium iodide (PI), or crystal violet [2]. The corollary was the failure of live cells to internalize supravital dyes, including neutral red, into lysosomes [3]. Therefore, by dual staining with trypan blue and neutral red, live and dead cells could be counted microscopically. Incorporation of [^{3}H]-thymidine or 5-bromo-2-deoxyuridine into newly synthesized DNA became a common indicator of cell proliferation in immunological and oncology

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Jeremy Simpson carried out his PhD at the University of Warwick, followed postdoctoral work at the Scripps Research Institute in San Diego, and the Imperial Cancer Research Fund in London. After 9 years as a staff member at the European Molecular Biology Laboratory (EMBL) in Heidelberg (Germany), he was appointed as professor of cell biology at UCD, in 2008. He currently applies high-throughput imaging technologies to study subcellular transport pathways and the internalization routes taken by nanoparticles in cells. He runs the UCD Cell Screening Laboratory and is the author of more than 80 publications.

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studies, respectively [4]. Leakage of lactate dehydrogenase (LDH) or potassium ions had an advantage of being more sensitive than dye exclusion or cell growth assays, because the latter are ineffective in acute toxicity studies as a result of insufficient proliferation during acute exposure. Trypan blue exclusion is an insensitive indicator of loss of cell viability, changing much later than LDH release, which in turn is less sensitive than the release of potassium and influx of sodium. This difference is likely to be attributable to the smaller size of an ion versus that of a large enzyme. Release of [^{51}Cr] from prelabeled cells became a common assay for the quantitation of cell-mediated cytotoxicity [5], although LDH release was more convenient, precise, and less expensive [6]. Many of the post-1980 assays focused on measuring the loss of reductive activity using dyes that were reduced in proportion to the activity of electron transport. Such assays are run on high-throughput, colorimetric or fluorescence-intensity, microtiter-plate readers, but crucially lack the ability to provide single cell resolution data. Thus, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was reduced by NADH generated by cell metabolism to a purple formazan precipitate, which reflects cytotoxicity, proliferation, or cell activation [7]. Other tetrazolium salts that yield a soluble formazan followed, along with the introduction of intermediary electron acceptors, such as phenazine methosulfate, to facilitate dye reduction [8]. The sensitivity of dye approaches was increased with a halving of assay time and reduction of costs by introduction of the Alamar Blue assay [9], which had a good correlation with neutral red uptake, LDH release, total protein, and cell density [10].

Other viability assays were developed that monitored changes in intracellular activity. Rhodamine-123 was the first mitochondrial membrane potential dye to become available and provided an earlier indicator of the loss of cell viability compared with trypan blue [11]. Relatively nontoxic, fluorescent dyes for the quantitation of intracellular ionized calcium were discovered and used to assess viability [12]. Given that there is a 10 000-fold gradient in free calcium concentration across the plasma membrane, a prolonged rise in intracellular calcium concentration indicates impaired cell health. For example, nonspecific membrane-perturbing agents, such as halothane, produce concentration-related increases in cytosolic calcium [13]. Many assays focus on the measurement of cell mass, which increases with cell growth and proliferation, but decreases with cytotoxicity. This effect can be quantified by fluorescent microtiter-based measurements of total DNA [14], total protein [15], or ATP content [16]. These and the reductive dye assays have greater throughput and ease of performance compared with the [^{3}H]-thymidine incorporation. Several cytotoxicity assays have been specifically developed for the assessment of apoptosis, including detection of plasma membrane annexin V, caspase activation, and shrinkage and fragmentation of nuclei. The implementation of sophisticated assays for screening during the 1990s resulted from the development and automation of multiwell, microtiter-plate high-content readers and the development of relatively nontoxic, subcellular, fluorescent dyes [17]. These sensitive assays screen overt and acute cytotoxicity and have a major role in drug discovery [13].

In vitro cytotoxicity and predicting drug attrition

A 7-year, international, multicenter study (MEIC) involving 29 laboratories and 61 different cytotoxicity assays evaluated the relevance of *in vitro* cytotoxicity testing in numerous cell types in relation to *in vivo* human toxicity [18]. Fifty chemicals were studied, including poisons, prescription drugs, substances of abuse, and common household chemicals. The *in vitro* cytotoxic concentrations were compared with known, acutely lethal doses in humans and showed predictive correlations of up to 88% [19]. The MEIC study suggested that assays with human cell lines give the best prediction and are independent of cell type, thereby indicating that the mechanism of toxicity was inhibition of a common, vital cell process. Other studies also provide further convincing evidence that *in vitro* cytotoxicity assays are highly predictive of acute human drug toxicity [20,21].

A retrospective study of concordance of human toxicity for 150 drug candidates in clinical trials was established and demonstrated close correlation with regulatory animal studies [22]. Human toxicity concordance was 71% for both rodent and nonrodent species, 63% for nonrodents alone, and 43% for rodents alone. Toxicity was identified in studies of duration of 1 month or less for 94% of toxicities. Concordance of animal and human toxicity varied depending on the type of toxicity: 91% (hematologic), 85% (gastrointestinal), 80% (cardiovascular), but only 37% (cutaneous hypersensitivity) and 55% (hepatic) for others. Even though prediction from preclinical animal models is especially low for human hepatotoxicity and cutaneous toxicity, when these occur, the candidate is likely to be terminated in clinical development despite the risk of a false positive. Although there is predictive value of regulatory animal studies for many human toxicities, drug safety remains an important cause of marketplace attrition. Of drug candidates entering clinical development from 2006 to 2010, ~25% failed because of safety issues [23]. Recognition of the high impact of safety-related attrition of drugs throughout all phases of discovery and development drove the implementation of novel cytotoxicity screens early in discovery to identify overt and acute toxicity [13]. Therefore, conventional cytotoxicity assays have been incorporated into drug discovery screening strategies because they use similar cell types, culture methods, and monitoring and measuring technologies as the other cell-based assays used to screen for appropriate efficacy or bioavailability. Additionally, preclinical development safety strategies typically include specific cytotoxicity assays for genetic toxicity using the *in vitro* micronuclei assay [24], and also when knowledge of the compound chemistry indicates specific risks, such as for phototoxicity using the neutral red uptake assay in 3T3 fibroblasts [25]. Unfortunately, these conventional screens have deficiencies in sensitivity and selectively, which impact the quality of prediction.

The HCA track record in drug discovery

The advent of HCA use in drug discovery *in vitro* toxicology over the past decade was an important departure in that it provide multiparametric mechanistic data in live cells in real time and this meant that, although animal toxicology was still needed to support first-in-man trials, there was greater confidence in achieving predictive outcomes with fewer false positives and false negatives. A cell-based HCA model demonstrated an order-of-magnitude increase in the concordance of *in vitro* cytotoxicity with human toxicity as part of an analysis of 250 marketed drugs over conventional cytotoxicity assays [26]. It focused in particular on low-incidence idiosyncratic hepatotoxicity. Since then, HCA has been

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