Cellular platforms for HTS: three case studies

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The field of cell-based screening is expanding rapidly as innovations in target selection and instrumentation increase the number of targets that can be efficiently screened in cellular formats. Cell-based screens can be configured to provide a broad range of data on chemical compound activity, mechanism of action and drugability. However, the decision to pursue a cell-based approach should not be made lightly, as cell-based assays can be challenging to implement in the high-throughput screening (HTS) laboratory. In this review, we describe three case studies in which targets were successfully interrogated in cell-based HTS, and highlight the necessary steps to ensure the validity of these screens.

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▼ HTS has become the dominant tool in the drug discovery process, partly because of the renaissance of one of the oldest approaches to lead generation: cell-based screening. In its earliest form, cell-based drug screening typically involved anti-microbial formats [1]. However, rapid progress in the fields of genomics and proteomics has greatly expanded the target landscape beyond microbial targets [1–6]. In parallel with these changes, combinatorial chemical synthesis has driven the production of large numbers of compounds with the potential for pharmacological activity [1–6]. The need to screen these large libraries of chemical compounds against multiple targets has stimulated improvements in instrumentation and automation that have evolved into the field of HTS [1–14]. Advances in assay technology and miniaturization have supported the evolution of multiple platforms for lead generation, including mammalian cell-based screens [2–14].

Upon selection of a target, a lead generation strategy is defined, which typically includes HTS as the first step (the 'primary' assay), followed by hit confirmation ('secondary' assays); these precede lead validation and optimization (Fig. 1a). Many factors can influence the format of the assays employed and their positioning in the screening paradigm, including the type of pharmacological information required for the target class, throughput, cost and other logistical or practical considerations. Although cell-based screens and biochemical or isolated target screens each have their merits [3,5–7], it is highly unlikely that any lead will progress to become a drug candidate without first having demonstrated activity in an appropriate cell-based model. Therefore, the question arises, where should the cell-based model be positioned in the lead generation process? Is the target amenable to a cell-based HTS, or is the throughput of the cell-based assays sufficiently limited as to warrant positioning later in the process? Some factors that influence this decision include: the cost and ease of target production, the validation of the target, the complexity of the target and the wealth of pharmacological information that can be obtained from a cell-based approach [3,5–8,11–14].

Target nature: cost and ease of production, validation state and complexity

If the isolation of functionally active enzyme or protein is technically challenging or expensive on the scale needed for HTS, a cell-based assay in which the cell becomes the expression factory could represent the fastest, least expensive approach [5,6]. There could also be intellectual property restrictions that limit the heterologous expression of a target that might be circumvented by screening the target endogenously expressed in a naturally derived cell line [6].

Likewise, cell-based HTS formats could represent the fastest approach to screening poorly characterized targets. The increased numbers of drug targets that are derived from genomics

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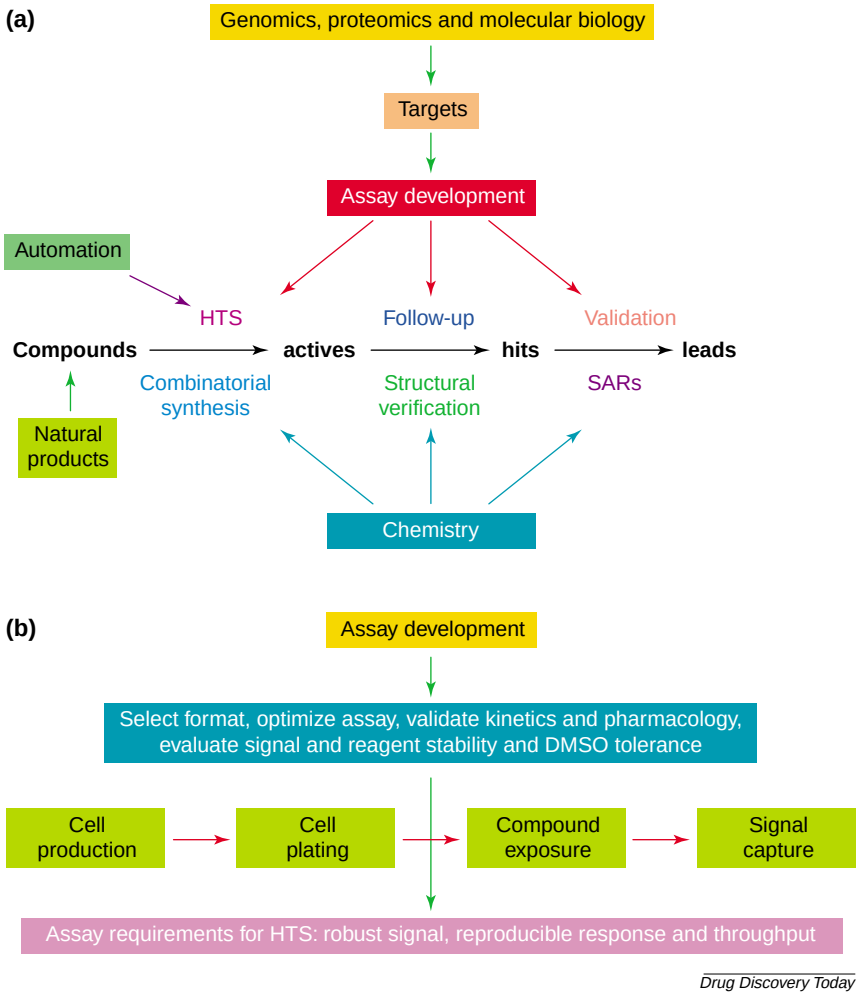

Figure 1. (a) Lead generation. Upon selection of a target, a lead generation strategy must be developed for primary and secondary assays for the confirmation of HTS actives, hit assessment, lead validation and lead optimization. **(b)** Cell-based assay development. Additional challenges to the development and implementation of cell-based screens beyond those associated with biochemical HTS formats.

approaches has driven the development of multiple 'gene to screen' paradigms to interrogate poorly defined targets, many of which rely on cellular assay systems [5,6]. For example, cell-based screening approaches have been heavily employed for orphan receptors with no known ligand [5,6]. These speculative targets are most easily screened in a format in which the target is expressed and regulated in the most physiologically relevant manner.

There are also complex targets that cannot be adequately configured or reconstituted in a biochemical assay [5,6,8,13,14]. Such targets could involve complex interactions between receptors, co-activators, co-repressors, response elements and other cellular factors that cannot be adequately reproduced outside the cell. These could include targets that regulate a signaling pathway, targets that are themselves regulated by poorly understood partners along a signaling pathway, or targets that require assembly of a transcriptional regulatory complex. Therefore, it might be best to screen such targets in a cellular system in which all of the necessary components are pre-assembled and regulated.

Wealth of pharmacological information

Cell-based functional assays can provide information on the nature of the pharmacological activity of a compound at a specific receptor, ion channel or intracellular target that cannot be obtained from a biochemical assay [5,6,13,14]. For example, cell-based functional assays provide a means to discriminate agonist, allosteric modulation and antagonist activity at receptors that cannot be determined in binding assays. Similarly, cell-based screening approaches provide information on the ability of the chemical compounds to penetrate the cell membrane, as well as an acute cytotoxicity profile. Several physiochemical, pharmacokinetic and toxicity factors impact drug efficacy and safety [15–17]. The pharmaceutical industry expends more money and resources on compounds that fail in development than succeed in the market place, and the major reasons for failure are toxicity and inappropriate kinetics [15–17]. Although the membrane permeability and cytotoxicity data obtained from cell-based assays cannot be considered definitive indicators of lead compound absorption or toxicity properties, they could serve to provide an alert for encumbered chemical series early in the lead generation process.

Our intent in this review is to describe generic requirements for cell-based HTS implementation, and discuss in detail the screen development process for three target classes that have been addressed in cell-based high throughput screens. The unique advantages and potential drawbacks of each screening format will be described, with an emphasis on the assay parameters that are crucial to successful implementation of a cellular assay in an HTS campaign.

Requirements for HTS

The purpose of HTS is the interrogation of large chemical collections in the context of a biological target to accurately identify active chemotypes. To achieve this purpose, screens must be configured to provide a robust, reproducible signal with adequate throughput to screen large compound libraries [18,19]. Because the initial activity of a compound in an HTS campaign will typically be determined in a single well at one concentration, the assay signal window (dynamic range) must be sufficiently rugged to provide adequate separation between the maximum and minimum responses, and should enable the response to active compounds to be discriminated from the background variability (noise) associated with the top and bottom of the signal window [18,19]. In addition to studies designed to validate the kinetics and pharmacology of the assay, efforts are made to optimize the signal window and/or variability of the assay in the context of several variables, including: dimethyl sulfoxide (DMSO) tolerance, reagent stability and signal stability (Fig. 1b). Cell-based screen implementation presents additional challenges, including: generation and/or characterization of an appropriate cell model, production of sufficient cells for HTS, plating cells for the assay, effects of compound exposure and capture of the assay signal (Fig. 1b).

Potential cellular 'platforms'

The choice of cellular platform, or background, has an enormous impact both on the development and implementation of the HTS for a target. The availability and behavior of the cells, together with the amplitude and reproducibility of the signal attainable against that cellular background, can all determine whether primary cells or cell lines, both native and engineered, are selected [5,6].

Primary cells of human origin are arguably the most physiologically relevant model system and several selected primary cell types, human and other species, are commercially available (e.g. Clonetics, Walkersville, MD, USA) and amenable to HTS [20]. However, in general, primary cells cannot be obtained at the scale necessary for HTS, and thus primary cell screens are positioned in the screening paradigm as low-throughput secondary assays.

Transformed cell lines of human origin are the most commonly used cell-based HTS platform. These lines can be screened in their native state for targets endogenously expressed in the cells, such as neuropeptide receptors expressed in SK-N-MC cells [21]. Many of these lines retain a highly differentiated phenotype and are an excellent platform on which to screen a complex physiological response, such as secretion; for example, insulin secretion from rat insulinoma (RIN) cells [22].

The advent of molecular and cell biology techniques to clone and express human proteins has provided access to cell lines with relatively high expression levels of the target of interest [23–25]. Cell lines can be engineered to express or over-express a target of interest. Expression can be transient or stable and several expression systems can be employed depending on the nature of the cell line and the target. Stable cell lines are most commonly generated by plasmid transfection or retroviral infection. Stable expression of the target is perhaps the approach of choice for HTS, but transient expression can be scaled up sufficiently to support a reasonable throughput.

Cell production and plating

To support an HTS campaign of weeks or months in duration, the cell culture must be scaled to support production of hundreds of microplates on a daily basis [5,6,15]. Cell culture media components and tissue culture hardware should be pre-tested and, where possible, single lots selected for the HTS. Logistical issues, such as cell viability, recovery from freeze-thaw, doubling times and cell yields must all be addressed during the development phase and monitored during the HTS. An optimal cell seeding density and the effects of the passage number or the growth phase of the cells on the assay signal should be determined because these can significantly affect the size of the cell bank required for the HTS.

One unique challenge to lower volume screening is cell seeding, particularly for adherent cells. This challenge is tied directly to the state of the art in liquid handling instrumentation, and will evolve as these instruments evolve [3,5,6]. The peristaltic action of the multidrop (Titertek Instruments, Huntsville, AL, USA) has provided an efficient method to deliver uniform numbers of viable cells to 96- and 384-well plates.

Compound exposure

Crucial challenges tied to cell-based screening, regardless of the assay format, include cytotoxicity, cell growth and the adherence properties of the cells. In cell-based HTS, compound cytotoxicity can mask activity at the desired target, producing false negatives, or it can produce false positives in inhibitor screens [5,6,15]. The degree of compound cytotoxicity depends upon the nature of the cell background, the compound dose and the length of exposure. Because most screening libraries are dissolved in DMSO, the DMSO tolerance of the assay can limit the maximum concentration of the compound that can be screened [5,6]. At 1% or greater, DMSO significantly interferes with most cell-based assays and, for some cells, tolerance might be significantly less.

In screens with long incubation periods (24–48 h), variations in cell growth across the plate can generate significant signal drift that usually manifests as edge effects. This differential growth is typically caused by temperature variations or media evaporation [3,5,6], and can be controlled by the use of plate lids, careful regulation of the humidity and temperature of the incubator chambers, and by ensuring that the microplates are distributed evenly in the incubators. Poor or inconsistent cellular adherence can also produce significant variation in the signal of certain assay formats. Poor adherence can be improved by coating the surface of the microplate with a charged substrate, such as poly-lysine, or with extracellular matrix components, such as fibronectin or collagen [5,6]. Coated plates are commercially available, or plates can be prepared in-house at a reasonable expense. During assay development, it is crucial to determine whether the assay could be simplified by eliminating or combining any of the aspiration or addition steps that might disturb the monolayer. It is also possible to adjust the volume, speed, height and angle of many liquid handlers to minimize the impact of the various manipulations on monolayer integrity.

Three case histories

Cells are capable of complex functions in response to a variety of stimuli, and many of these could be configured to provide an assay format suitable for HTS or lead generation: gene transcription, ion flux, transport, proliferation, cytotoxicity, secretion, translocation, redistribution, protein expression and enzyme activity. We have selected three common assay formats to showcase some of the issues involved in cell-based screening. Approximately half a million compounds were screened in each of the three assay platforms described.

First case history: promoter-driven luciferase reporter

In the 1980s, molecular and cell biologists used reporter genes to investigate the 5' untranslated regions of cDNAs to determine which sequences were involved in modulation of gene transcription. In the ensuing decades, the technology and instrumentation have evolved such that many target classes are now amenable to cell-based HTS in reporter formats: G-protein-coupled receptors (GPCRs), receptor kinases and nuclear receptors [5,6,11,26–36]. Reporter assays couple the biological activity of a target to the expression of a variety of readily detected enzymes or proteins: chloramphenical transferase, firefly and renilla luciferase, secreted human growth hormone, β-galactosidase, secreted alkaline phosphatase, green fluorescent protein(s) and β-lactamase [6,11,27]. Reporter enzymes provide a highly amplified signal, thereby providing sensitivity, and luciferase appears to be the most commonly used reporter enzyme for HTS [6,11]. Transcriptional regulation assays are configured by linking the natural promoter, or elements of the promoter, of the gene of interest to the coding region of the reporter gene [26,27,34]. To take advantage of common signal transduction pathways, synthetic repeats of a particular response element can be inserted upstream of the reporter gene to regulate its expression in response to signaling molecules generated by activation of that pathway [26–36].

The case history that we have selected for discussion involves a promoter-driven luciferase reporter stably expressed in a mononuclear phagocyte cell line (Fig. 2). Although there is a clearly discernable background level of luciferase expression in untreated cells, overnight treatment with the appropriate agonist produced a significant, and dose-dependent increase in the level of luciferase expressed (Fig. 2a and 2b). At maximum agonist doses, the luciferase induction averaged between 4–6-fold over background, and was sufficiently robust and reproducible that a seeding density of 2.5×10^3 cells per well was selected for the HTS. The assay tolerated up to 1.4% of DMSO without significant impact on the signal window (Fig. 2c). For the HTS, compounds were diluted and run at a final DMSO concentration of 0.5%. The agonist dose response of the optimized assay was extremely consistent (Fig. 2b).

The active rates for an HTS will be determined by both the concentration of the compounds tested, and the active criterion selected for the screen. During assay development, the robustness of the assay signal window and the variability (noise) associated with the top and bottom of the response will be used to establish an active criterion for the HTS. As discussed previously, compounds that meet or surpass this criterion should therefore have a good probability of being a real active in the assay. For our promoter-driven luciferase reporter, the assay signal window produced a Z-factor of 0.574 (Fig. 2d), which indicates that it is an excellent assay for HTS. Data from two plates treated with a 50% agonist dose were included in the assay signal window experiment (Fig. 2d). These data illustrate that for compounds that induce 50% activation of the reporter, there is a high probability that they will be real activators. The initial active criterion for the definition of an HTS active was set at 50% activation of the reporter; however, the data were sufficiently reproducible that activation in the 30–50% range would also probably be significant. Although the HTS was run in 384-well plates, the assay was also easily adapted to 96-well and 1536-well formats (data not shown). In HTS, approximately 0.14% of the compounds screened met the active criterion, and of those selected for dose response confirmation, 33.6% produced dose-dependent

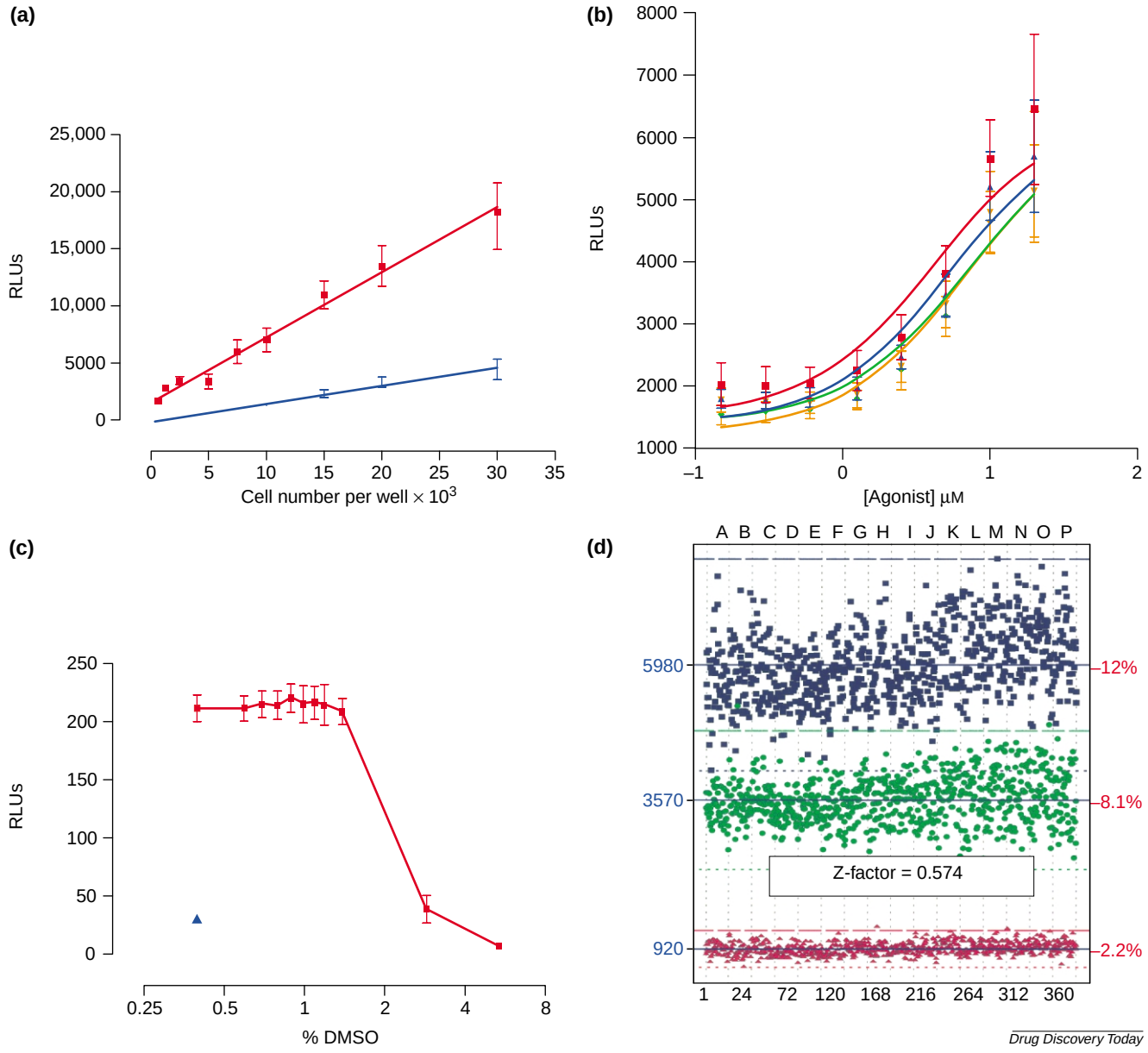

Figure 2. First case history: promoter–luciferase reporter assay, 384-well format. Here, 20 μl of compounds and controls were added to 384-well Costar (Corning Costar, Cambridge, MA, USA) white opaque tissue culture plates using a 384-well stacking MultiMek (Beckman Coulter, Schaumburg, IL, USA), and 20 μl of viable cells stably expressing the promoter-driven luciferase reporter were seeded into plates containing compounds using a 384-well stacking Multidrop (Titertek Instruments; Huntsville, AL, USA). Plates were incubated for 20–28 h at 37°C, 95% humidity and 5% CO_2. The luciferase activity of the cells was measured using the Northstar imaging plate reader (PE Biosystems, Foster City, PA, USA) operated in the inject and read mode; 30 μl of Bright Glo™ luciferase reagent (Promega, Madison, WI, USA) was injected per well and plates were read for 30 seconds. **(a)** Cell seeding density effects: red = agonist, blue = unstimulated; **(b)** Agonist EC_{50} curves run on four separate days: red = day one, blue = day two, green = day three, orange = day four; **(c)** Dimethyl sulfoxide (DMSO) tolerance: red line = induced, blue triangle = non-induced; and **(d)** Assay signal window and reproducibility, two plates each of a maximum, minimum and mid-point response.

induction of luciferase. The remaining 66% of the HTS actives failed to confirm in dose response assays.

Widespread implementation of reporter assays has driven the development of multiple instrumentation platforms, and commercial reagent kits have been optimized to provide greater signal amplitude or stability [26–36]. The development of low volume liquid handling capability and charge-coupled device (CCD) imaging plate readers has enabled reporter screens to be miniaturized into higher density formats, such as 384- and 1536-well plates [28]. However, gene expression assays are subject to non-specific interference by compounds that act distally to the target,

or when an inhibitor activity is the desired profile, cytotoxic compounds might appear as false actives [5,6,11]. Cytotoxicity is a common secondary assay format used to triage the inhibitor actives from a cell-based HTS, and several radioactive and non-radioactive assay kits to quantify cell proliferation and cytotoxicity are commercially available [6,15]. Target selectivity issues for reporter assays could also be addressed by running the same reporter gene, driven by other promoters, in the same or varying cell backgrounds [26,27].

Although reporter assays have been successfully applied to GPCR targets [29,31–33,35,36], the delay in signal development, typically 4–5 hours, that is inherent in the reporter gene system could make this approach less attractive for the screening of rapid cellular responses. One could argue that this indirect and amplified signal might obscure subtle modulation of the receptor. For example, reporters that are designed to detect Gs-coupled receptor activity are often dependent on amplification of the signal by multiple copies of the cyclic adenosine monophosphate (cAMP) response element. This amplification can result in unusual concentration-dependent responses [37]. Reporter assays designed to detect the activity of receptors that signal via mobilization of calcium typically use the nuclear factor of activated T cells (NFAT) response element. However, NFAT-mediated gene expression is dependent on both the amplitude and duration of the calcium flux and, therefore, might not be useful to detect rapid calcium-mediated responses [38].

Second case history: GPCR FLIPR™ Ca^{2+} response

It might be desirable to design screens to detect signaling events more proximal to a receptor; for example, GPCR activation can be detected by direct measurement of the receptor-mediated cAMP accumulation, or changes in intracellular Ca^{2+} concentration. GPCR targets that couple via G_q produce an increase in intracellular Ca^{2+} that can be measured in HTS using a combination of a calcium-sensitive dye, such as Fluo-3 or calcium green, and a fluorescence plate reader [39–44]. The second case history looks at a $G_{I/o}$-coupled receptor that was configured for a 384-well assay on the fluorescence imaging plate reader (FLIPR™, Molecular Devices, Sunnyvale, CA, USA). A stable cell line was generated expressing both the receptor and a promiscuous ($G\alpha_{15}$) G-protein to 'switch' receptor activation to an increase in intracellular calcium. The FLIPR™ has a cooled CCD camera imaging system, which collects the signal from each well of a microplate (both 96 and 384-well) simultaneously [39]. The FLIPR™ can read at sub-second intervals, which enables the kinetics of the response to be captured, and it has an integrated pipettor that can be programmed for successive liquid additions. These capabilities provide an opportunity to investigate subtle effects on the receptor, such as discrimination of agonist, allosteric modulation and antagonist activity within the same well [40–44].

Cells lacking either the receptor or promiscuous G-protein failed to elicit a detectable Ca^{2+} response when treated with agonist (data not shown). Agonist stimulation produced a significant, dose-dependent increase in Fluo-3 fluorescence relative to the background signal from unstimulated cells (Fig. 3a and 3b). The effects of cell seeding density on the assay signal window are shown in Fig. 3a. A seeding density of 2000 cells per well produced a robust and reproducible response (Fig. 3a, 3b and 3d) and was selected for the HTS. Cell plates could be used after 24, 48 and 72 hours of tissue culture post-seeding (data not shown). Although the CCD camera collects the signal from each well of a microplate simultaneously, thereby providing adequate throughput in 384-well format, the optics of the device limit fluorescence detection to the bottom of the well. This reduces background fluorescence, but also requires that the cells be firmly attached to the plate bottom. Dye loading is a multi-step process of aspirations and buffer additions that can be disruptive to cell monolayers and, in our experience, is not very amenable to automation. The height and speed of the FLIPR™ pipettor, and the volume of addition, all need to be adjusted to minimize the impact on monolayer integrity. Microtiter plate characteristics can dramatically affect the response and, for many cell types, coated plates are an absolute requirement. Selection of an optimal cell-seeding density matched to a particular plate type, definition of a dye loading process and determination of the FLIPR™ control settings are the major challenges for assay development. In many cases, the best signal is obtained from densely seeded wells, which can lead to large cell culture requirements for the implementation in HTS.

At DMSO concentrations greater than 2%, there was a dose-dependent increase in the Fluo-3 fluorescence signal from unstimulated cells that collapsed the assay signal window (Fig. 3c). For the HTS, compounds were diluted such that the final concentration of DMSO was 0.5%. The agonist dose response of the optimized assay was extremely consistent (Fig. 3b), and the assay signal window produced a Z-factor of 0.511 (Fig. 3d), which indicates that it is an excellent assay for HTS. Data from two plates treated with a 50% agonist dose were included in the assay signal window experiment, to mimic the activity of an agonist compound (Fig. 3d). These data illustrate that any compound that induces 50% activation of the Ca^{2+} response would have a good probability of being a real agonist.

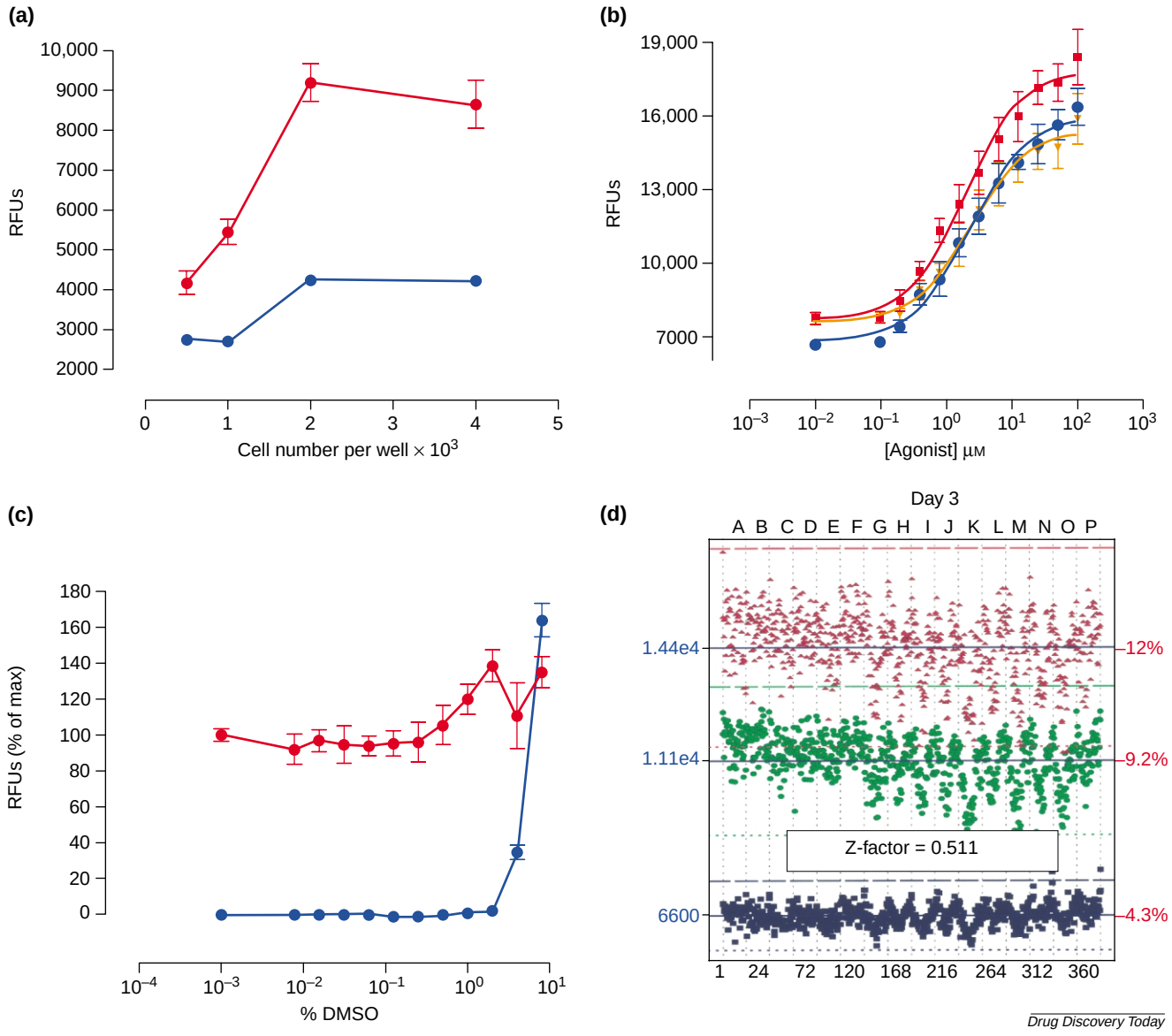

Figure 3. Second case history: G-protein-coupled receptor (GPCR) target, fluorescence imaging plate reader (FLIPR™, Molecular Devices, Sunnyvale, CA, USA) Ca^{2+} response, and 384-well format. Cells stably expressing both the receptor and a promiscuous ($G\alpha_{15}$) G-protein were seeded into 384-well black clear-bottom plates (Corning Costar; Cambridge, MA, USA) using a 384-well stacking Multidrop (Titertek Instruments, Huntsville, AL, USA). Plates were incubated for 24, 48 or 72 h at 37°C, 95% humidity and 5% CO_2. Tissue culture media was removed from the plates by inversion, and 20 μl of 8 μM Fluo-3 dye (Molecular Probes, Eugene, OR, USA) diluted in Hank's balanced salt solution (HBSS) (Biowhittaker; Rockland, ME, USA) was added to each well using a Platemate (CyBio, Jena, Germany) and plates were incubated at 25°C for 120–240 min. The dye was removed from the plates by inversion and 20 μl of HBSS buffer was added using a 384-well stacking Multidrop. Cell plates, test compound plates diluted in HBSS, and agonist plates were placed on the deck of the FLIPR™, a laser light check was performed on the cells, and the first (test compounds) and second (agonist) additions were performed at the required times by the integrated 384-well pipettor. **(a)** Cell seeding density effects: red = buffer; blue = agonist; **(b)** Agonist EC_{50} curves run on three separate days: red = day one; blue = day two; orange = day three; **(c)** Dimethyl sulfoxide (DMSO) tolerance: red = ligand, blue = buffer; and **(d)** Assay signal window and reproducibility, two plates each of a maximum, minimum and mid-point response.

However, the initial active criterion for the definition of an HTS active was set at 70% activation of a maximal agonist-induced Ca^{2+} response. The ability of the FLIPR™ to make two additions to the cells enables us to detect agonists and antagonists, in one assay. The unknown compound to be tested is added by the FLIPR™ pipettor in the first addition. A significant Ca^{2+} response, in our case >70% of the maximal response, in the first read indicates that the compound might be an agonist. The second addition consists of a ~90% maximal concentration of known agonist and a

significantly reduced response to agonist, in our case <70%, indicates that the compound might be an antagonist. In HTS, 0.25% and 0.5% of the compounds screened met the active criterion set for agonists and antagonists, respectively. Of the putative agonists and antagonists selected for dose response confirmation, 56.6% of the agonists tested produced dose-dependent agonist activity, and 5% of the antagonists tested produced dose-dependent antagonist activity. After reviewing the antagonist data, it seemed probable that the majority of false positives were from wells that failed to receive the control agonist in the FLIPR™ second addition during the HTS.

Although the FLIPR™ has facilitated advances in cellular calcium mobilization screens, these assays remain difficult to configure, are relatively slow and are fraught with potential artifacts. Blocked FLIPR™ tips will lead to false positives in an inhibitor screen or false negatives in an agonist screen. Fluorescent compounds, Ca^{2+} ionophores and compounds that permeabilize the cell membrane can all contribute to false positives in the agonist read and could also interfere with antagonist reads. These types of nuisance or interference compounds can often be identified from the kinetic traces of the response, but this kind of in-depth data review is time-consuming and requires experience to correctly recognize strange response profiles. The use of the FLIPR™ and calcium dye approach for screening GPCR targets has been greatly enabled by the over-expression of promiscuous ($G\alpha_{16}$ and $G\alpha_{15}$) and chimeric ($G\alpha_{qi5}$ and $G\alpha_{qo5}$) G-proteins, which provide a method of 'switching' $G_{I/o}$-coupled receptor activation to an increase in intracellular calcium [44]. However, screens that are designed to detect receptor activity against a backdrop of stable, high-level promiscuous G-protein expression are also susceptible to artifacts – false positives presumably derived from other cell surface receptors that hijack the promiscuous G-proteins. Indeed, even in the absence of a promiscuous G-protein, any endogenous GPCR that couples through Gq and induces a Ca^{2+} response might show up as an agonist, or interfere with antagonist reads. It is well documented that GPCRs, particularly those in heterologous expression systems, can activate multiple signal transduction pathways [24,42], and indeed there is also evidence for cross-talk between recombinant and native receptors that could also complicate the responses to compounds [42]. One strategy to triage false positives is to run a secondary screen against the parent cell line that lacks the receptor of interest.

Third case history: transporter–radioligand uptake

Cell-based screens for transporter targets, such as amino acid or neurotransmitter carriers, are preferred when ligand binding to the transporter is complicated by either the high nonspecific binding of 'sticky' ligands, or low expression of the transporter. Cell-based approaches to transporter targets enable identification of allosteric modulators that could be missed in a binding assay. We have selected a carrier-mediated transport target, endogenously expressed in a rat basophilic leukemia cell line, and run in a Cytostar-T™ (Amersham Pharmacia Biotech, Little Chalfont, UK) format assay as our third HTS case history (Fig. 4). Cytostar-T™ scintillating microplates, an adaptation of the scintillation proximity assay (SPA) technology, are 96-well tissue culture-treated plates in which the transparent base of the plate is impregnated with scintillant that produces light when a radiolabeled substrate of suitable decay characteristics is brought in close proximity to the base [8,45–48]. Radioactive substrates that bind to the cell, or which are taken up by the cell via ion channels or transport carriers, will produce light in proportion to the amount of radiolabel present [8,45–48]. Cytostar-T™ plates have been used to measure the influx or efflux of radiolabeled tracer ions appropriate for the ion channel target of interest [8,46,47]. In addition, the Cytostar-T™ technology has been used to configure cell-based assays for the uptake of radioactive substrates, cell cycle synchronization, cell proliferation and cytotoxicity [45,48].

The amount of radioligand accumulated by the cells was dependent upon the number of cells seeded in the wells, the concentration of radioligand and both the time and temperature of incubation (Fig. 4a and 4b). Non-specific uptake of the radioligand was estimated in the presence of 100 μM of a known inhibitor of the carrier. Although the level of uptake at ambient temperature was significantly reduced when compared with levels in cells incubated at 37°C (Fig. 4b), the assay signal window was still sufficiently robust that the HTS was conducted at ambient temperature. We have observed similar effects of temperature on the uptake of all the carrier-mediated transporter targets we have evaluated to date. The operational benefits of being able to conduct the uptake assay at ambient temperature substantially outweigh the relatively minor increase in K_m that was observed. The assay tolerated up to 2% of DMSO without significantly impacting the signal window (Fig. 4c), and for the HTS, compounds were diluted and run at a final DMSO concentration of 0.5%. The assay signal window produced a Z-factor of 0.847 (Fig. 4d), which indicates that it is an excellent assay for HTS. Data from two plates treated with an 80% dose of inhibitor were included in the assay signal window experiment to mimic the activity of an inhibitor compound (Fig. 4d). As the data illustrate, any compound that induces 80% inhibition of the uptake response, would have a good probability that

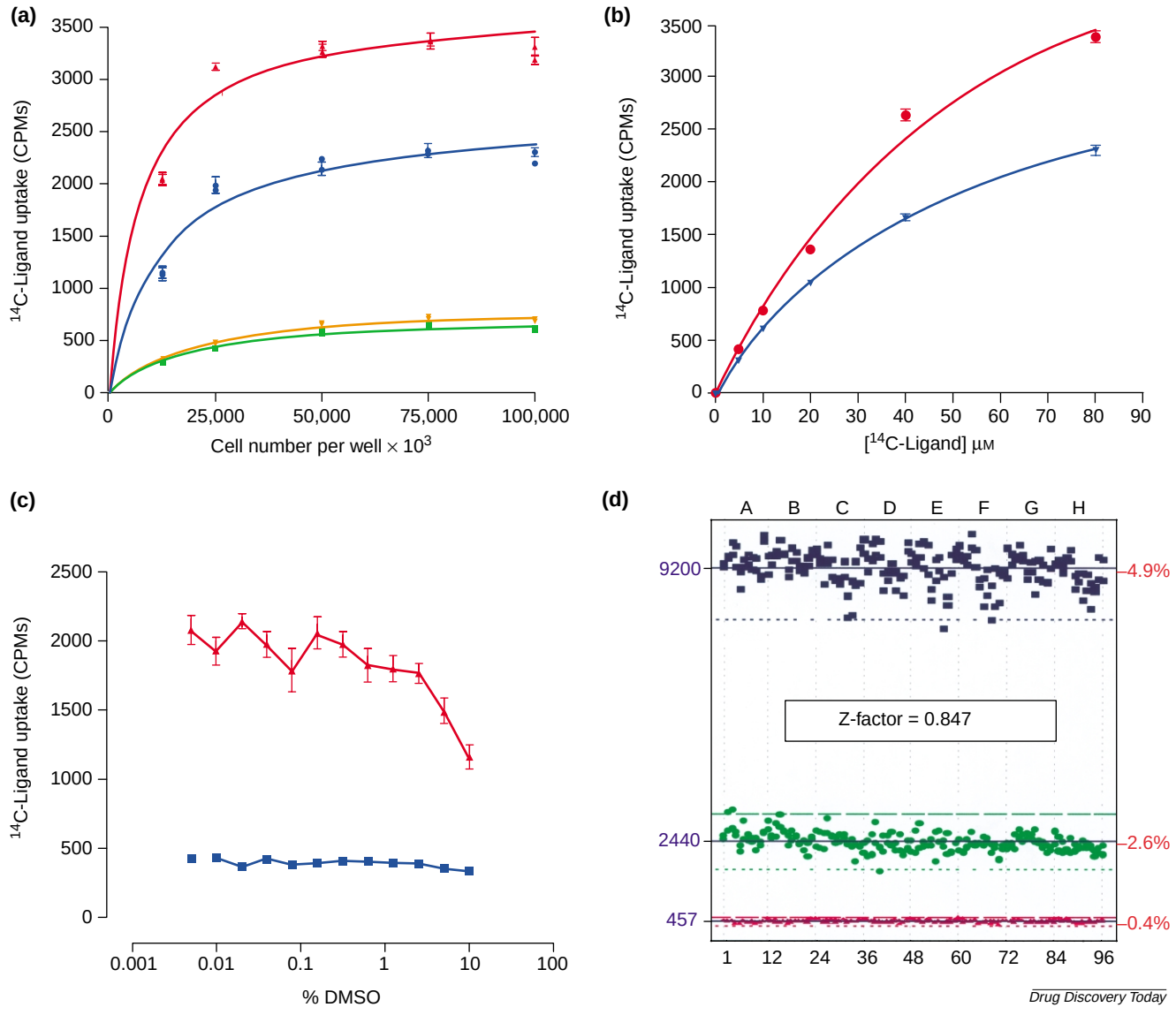

Figure 4. Third case history: transport target, Cytostar-T™ format (Amersham Pharmacia Biotech, Little Chalfont, UK), and 96-well format. Rat basophilic leukemia cells were seeded into the 96-well Cytostar-T™ plates using a 96-well stacking Multidrop (Titertek Instruments, Huntsville, AL, USA). Plates were incubated for 24 h at 37°C, 95% humidity and 5% CO_2. Media was removed from the cells and replaced with 100 μl of uptake buffer (25 mM HEPES, 125 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_47H_2O$, 5.6 mM glucose, pH 7.4) using an SLT aspirator/dispenser (SLT Instrumentation, Research Triangle Park, NC, USA). A 96-well stacking MultiMek (Beckman Coulter; Schaumberg, IL, USA) was used to add 50 μl of test compounds and plate controls in uptake buffer to the plates, 50 μl of ^{14}C-substrate in uptake buffer was added to the plates using a 96-well stacking Multidrop, plates were sealed using a plate sealer (Zymark; Hopkinton, MA, USA), incubated at ambient temperature for the required time (typically 60–90 min), and then read on a Microbeta® counter (Perkin Elmer Wallac; Gaithersburg, MA, USA). **(a)** Cell seeding density and time of incubation effects: red = 120 min, total uptake (counts per minute; CPM); blue = 60 min, total uptake (CPM); orange = 120 min, non-specific uptake (CPM); green = 60 min, non-specific uptake (CPM); **(b)** Substrate saturation curve and temperature effects: red = 37°C, blue = ambient temperature; **(c)** Dimethyl sulfoxide (DMSO) tolerance: red = total uptake (CPM); blue = non-specific uptake (CPM); and **(d)** Assay signal window and reproducibility, two plates each of a maximum, minimum and an 80% inhibitor response. Non-specific uptake refers to the amount of uptake measured (CPM) in the presence of 100μM of a known inhibitor of that transporter.

it is a real inhibitor. The initial active criterion for the definition of an HTS active was set at 50% activation of uptake, and 2.34% of the compounds tested in HTS met that criterion. Of those selected for dose response confirmation, 60% produced dose-dependent inhibition of uptake.

The Cytostar-T™ format requires that the cells are firmly attached to the plate bottom and, for loosely adherent cell types, coated plates are an absolute requirement. The height, speed, angle, position and volumes of all automated liquid handling steps need to be adjusted to minimize

the impact on monolayer integrity. As with any cell-based HTS looking for inhibitors, acutely cytotoxic compounds might show up as false positives. In general, however, transport assays are relatively short assays (60–90 min), such that with the limited compound exposure times and by controlling the dose of test compounds, the impact of cytotoxic compounds can be limited. As discussed above, there are many cytotoxicity assay formats that are compatible with HTS and which can be used to triage actives. Compounds with detergent-like properties that permeabilize the cell membrane can also show up as false positives. Compounds that behave like ionophores, or interfere with the membrane potential and/or the normal homeostasis of ion distribution within the cell, could also behave as false positives if the uptake mechanism requires co-transport of ions to drive the process. One strategy to triage false positives and evaluate target selectivity is to run a secondary screen against another carrier target, preferably from the same transporter family and in the same cell background. Cost has limited the application of the technology in HTS because there is a substantial access fee associated with the SPA and Cytostar-T™ technology and plates are relatively expensive.

Conclusions

Across the industry the number of targets addressed in cell-based high-throughput screens is increasing. This trend has been facilitated by advances in screening technology and by the recognition that the challenges of cell-based screen implementation can be offset by the additional data extracted from these approaches. In cells, the activity of a target is regulated under 'physiological' conditions, so that cell-based formats can be configured to screen a specific phenotype in a system capable of complex functions. Cell-based functional assays can therefore provide information on the nature of the pharmacological activity of a compound that cannot be obtained from a biochemical assay. Likewise, cellular formats generate data on compound cytotoxicity and cellular membrane permeability that could serve to provide an alert for encumbered chemical series early in the lead generation process.

Recent advances in assay technology, instrumentation and automation have facilitated an expansion in mammalian cell-based screening at all stages of the lead generation process. In the case histories presented, a cell-based assay format was selected as the primary HTS for three distinct target classes, and screening campaigns were successfully prosecuted for each target against a large chemical library. Each primary screen exhibited a robust signal window and reasonable variability with correspondingly good Z-factors. Though the formats of the three screens were quite different, each was successfully employed to identify active chemotypes. All three screens yielded compounds that progressed further in the lead generation paradigm.

Clearly, cell-based assay formats can be scaled successfully for HTS. However, the decision to pursue a cell-based approach for primary screening must be considered for each target. As more poorly characterized targets enter the screening process, the ability to rapidly address these targets in a high-throughput cellular screen becomes crucially important. Likewise, it becomes necessary to implement cellular secondary assay panels to rapidly de-convolute compound activities observed in the HTS, and to address cytotoxicity and membrane permeability issues.

The instrumentation coming to the HTS market suggests that the trend in cell-based screening is towards further complexity; that is, higher content formats as captured by the Arrayscan instrument (Cellomics, Pittsburgh, PA, USA) and others [13,14]. As these instrumentation platforms evolve, the number and complexity of targets that can be addressed in cell-based HTS will expand, suggesting that the field of cell-based HTS will continue to be a focus of innovation in the drug discovery process.

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