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Recent developments in the supply of cells for screening and new assay technologies are changing the view that working with living cells is labour intensive and 'a little bit tricky'.

Cryopreserved cells facilitate cell-based drug discovery

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Advances in detection technologies have enabled an increased use of cell-based functional assays in early drug discovery, in particular for G protein-coupled receptors. Screening assays that use live cells are less prone to generate false positives than assays using lysed cell samples. The use of cryopreserved cells instead of cells that are continuously maintained in culture decreases day-to-day variation, removes passage effects and improves the consistency of cell-based assay results. Cryopreservation techniques uncouple cell culturing from drug-screening activities and allow the use of cells as reagents, just like enzymes in biochemical assays.

It has long been recognized that live cells are a closer mimic of *in vivo* systems than purified proteins or membrane preparations. However, working with living cells has always been considered to be labour intensive and difficult. Recent developments in the supply of cells for high-throughput screening (HTS) are changing this view, while new assay readouts and approaches for hit deconvolution further improve the efficiency of HTS.

Modern drug discovery is a highly industrialized process in which the chance of finding good starting points for drug development is enhanced by screening large numbers of target biomolecules against large collections of unique chemical compound libraries. Although *in vitro* biochemical assays, such as enzyme activity and receptor binding, have been used extensively in the past, there is a rapid increase in the use of assays based on living cells. Cell-based assays provide a target in a more physiologically relevant environment than biochemical assays. According to a worldwide survey involving more than 50 pharmaceutical and biotech companies, 53% of all primary screens were anticipated to be cell-based in 2006, as compared to 46% in 2004 [1]. Although novel assay technologies and approaches for hit deconvolution improve the efficiency of HTS, screening is becoming more flexible because of the increasing availability and validated use of cryopreserved and division-arrested cells.

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Cell-based assay technologies

Cell-based assays are predominantly used to screen targets that are refractory to biochemical purification, such as G protein-coupled receptors (GPCR). GPCRs are the most successful single drug target class, as shown by the number of marketed medicines interacting with these proteins. Analysis indicates that one fourth of prescription drugs sold worldwide exert their therapeutic effect through approximately 70 different GPCRs [2]. GPCRs are also the most popular target type in HTS. According to a worldwide survey, on average one third of all primary screens are prosecuted against this target type [3]. Traditionally, GPCR modulators have been identified with displacement assays using radiolabelled ligands and membranes expressing the receptor of interest. In the past few years, many new cell-based screening technologies have become available [4]. These technologies take advantage of the functional responses elicited following GPCR activation. Coupling to $G\alpha_s$ and $G\alpha_i$ proteins results in an increase or decrease in intracellular cAMP concentration. Coupling to $G\alpha_q$ proteins results in the mobilization of intracellular calcium. There are several HTS assays to measure cAMP levels in lysed cell samples [5,6]. These assays use different fluorescent or chemiluminescent readouts and have different sensitivities [6], but they are all derived from the same principle of a labelled form of cAMP competing with the endogenously produced cAMP for binding to anti-cAMP antibodies. Transient calcium fluxes can be measured in live cells with

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calcium-sensitive dyes in a fluorescence imaging plate reader (FLIPR) [7] or with recombinantly expressed calcium-sensitive photo proteins, such as aequorin [8] or Photina® (http://www.axxam.com). The effect of G protein activation can also be measured further downstream in the cell with transcriptional reporter genes, such as luciferase or β-lactamase [9–12].

GPCR signalling is attentuated by phosphorylation of the intracellular loops of the receptor by GPCR kinases, followed by binding of β-arrestin. The internalization of GPCRs by β-arrestin provides a G protein subtype independent readout and can be used for screening of receptors whose signalling mechanism is unknown [13]. Receptor internalization and β-arrestin recruitment can be visualized by creating chimeras with green fluorescent protein (GFP) and high-content cellular imaging [13,14].

Cell-based assays are also applied for the screening of ion channels and protein kinases. The activation of calcium-permeable ion channels can be measured using ion-sensitive probes and FLIPR, similar to $G\alpha_q$ protein-coupled GPCRs [15]. Voltage-sensitive ion channels can also be probed in living cells using dyes that are sensitive to changes in membrane polarization [16]. Protein kinases are usually screened with biochemical assays based on proteins expressed in insect cells or bacteria [17,18]. Growth factor receptor tyrosine kinases are typically screened with enzyme assays in which only the kinase domain, and not the complete protein, is used. This approach works well for the identification of antagonists, but agonists or growth factor mimetics can be found only with cell-based assays. Activation of growth factor receptor kinases can be measured in cell lysates with anti-phosphotyrosine antibodies [19,20].

Successes from cell-based screening

There are two major advantages of employing cell-based functional assays over ligand-binding assays in primary HTS [12,21]. Firstly, cell-based assays have improved sensitivity for agonism caused by the intracellular signal amplification and allow identification of both agonists and antagonists. Secondly, they allow for the identification of compounds with diverse pharmacological effects. Compounds can act through the same binding site as the natural ligand (orthosteric ligands), while compounds that act at another site in the receptor and function as allosteric modulators may also be identified in a functional screen [22]. Cinacalcet (Sensipar™) is an allosteric enhancer of the calcium-sensing receptor and a drug for the treatment of hyperparathyroidism in patients with chronic kidney disease [23]. Repertaxin is an allosteric inhibitor of the chemokine receptors CXCR1 and CXCR2 and is in clinical trials for reperfusion injury [24]. At Organon BioSciences (Oss, The Netherlands) low molecular weight agonists of the luteinizing hormone (LH) and the follicle-stimulating hormone (FSH) receptors have been discovered by screening a library of synthetic chemical compounds with a cAMP-dependent luciferase reporter gene assay [25,26]. As the natural ligands of the LH and FSH receptors are very large dimeric glycoproteins, it would have been impossible to predict the structures of these small molecule mimetics on the basis of pharmacophore models. While the natural hormone ligands interact with the large extracellular domain of the receptors, the small molecule drugs are thought to bind in an evolutionary conserved pocket in the seven-transmembrane domain, where they act as allosteric modulators [25–27]. These success stories demonstrate the utility of cell-based HTS for drug discovery.

De-selection of screening artefacts

One of the main challenges of cell-based screening is to discriminate between true hits and artefacts. After the primary HTS, the activity of hits is confirmed by re-testing in the same assay to remove false positives caused by the statistical variation of the assay signal. The number of false positives may comprise up to several percentages of compounds screened, depending on the cut-off set to assign a compound as a hit and on the quality of the assay. To discriminate further between true hits and artefacts, additional assays are performed. Autofluorescent and coloured compounds may interfere with fluorescent or chemiluminescent probes and cause false positives. These technology artefacts can be removed by applying a secondary assay with a different readout [12]. Compounds that interfere non-specifically with signal transduction may also appear as artefacts but can be removed by screening against a cell line lacking (over)expression of the target of interest. Another way to remove false positives is by counter-screening against a different target expressed in the same cell line, for example, against the ATP receptor P2Y1 to remove compounds interfering non-specifically with calcium signal transduction [28]. These compounds may be removed from the library or considered as starting points for chemical genomic approaches.

Once defined as a true hit, a compound's selectivity is determined by testing against related targets, preferably expressed in the same cellular background. After several rounds of chemical modification and improvements in potency and selectivity, a lead compound is derived. During lead optimization compounds are usually tested in several cell-based assays in parallel. This allows improvements in potency to be monitored simultaneously with improvements in selectivity.

Pitfalls of cell-based HTS assays

Assay technologies originally developed for HTS are now also widely applied in lead optimization programs. As these cell-based assays move more downstream in the drug discovery process, they may create new pitfalls. Receptor expression level and coupling efficiency can affect the potency of standard agonists in cellular assays. Overexpression may increase a receptor reserve, leading to a leftward shift of the dose–response curves of agonists, and partial agonists to become full agonists. While the assay becomes more sensitive for HTS, compound activity may be overestimated.

Different technologies for cell-based assays also have their specific limitations that should be taken into account. Luciferase reporter gene assays are generally robust and sensitive and have been applied successfully to identify receptor agonists [25,26]. However, in antagonistic screens luciferase is much more prone to false positives than the β-lactamase reporter gene technology [11,12]. To measure luciferase activity, cells are lysed. In contrast, β-lactamase is measured in living cells. In cAMP assays, it is important to convert signal response into cAMP concentration using a cAMP standard curve, to produce the most relevant estimate of compound potency and efficacy [5]. In contrast to reporter gene assays and cAMP, calcium flux assays are non-equilibrium assays. Compounds with a relatively slow association rate may be missed in a protocol with short incubation time [7]. Overexpres-

sion of the promiscuous $G\alpha_q16$ protein [29] has been used to force GPCR signal transduction towards calcium readouts and provides a non-physiological readout. Forced coupling can be of use in HTS to detect initial hits but may distract from real pharmacological relevance in target validation and lead optimization studies.

Division-arrested cells

Cells are live and dynamic reagents. Instability of target protein expression, cell passage number, growth phase and differences in cell handling can cause significant assay variability. HTS requires very large batches of living cells just in time. Routine cell culture maintenance of adherent cells and plate preparation for screening can be automated with robotic systems such as SelecT from the Automation Partnership (http://www.automationpartnership.com/). However, because of the high cost of equipment, these solutions may only be cost effective in large screening laboratories [5,7].

After growing in appropriate amounts, cells are usually applied immediately in an assay. Quality is verified during the screen, for example with dose–response curves of reference compounds. If problems with the quality of cells are not discovered before a large screening campaign, significant amounts of time, reagents and compounds may be wasted. A simple deviation from standard practice is the use of cryopreserved cells. Instead of maintaining cell lines continuously in culture while performing a screen, cells are grown in one large batch and then stored at very low temperature (e.g. −140 °C) [30,31]. To validate cell quality, a small batch is pharmacologically tested, while the remaining lot is defrosted when needed for a screen. If the validation is satisfying, this is then followed by a large scale up of cells, frozen down in batches. One of these batches is then defrosted for a confirmational test.

For fully automated screening platforms, processing ten thousands of compounds per day, there can be a considerable time-lag between processing of the first and the last assay plate. Because cell doubling may cause significant plate-to-plate variability in assay signal, cells for HTS are usually maintained and suspended in serum-free medium. In addition, cells can be treated with mitomycin C to arrest mitosis [32–34]. Mitomycin C is a natural antitumor antibiotic and cytotoxic drug used in clinical chemotherapy for the treatment of various carcinomas [35]. The cytotoxicity of mitomycin C is primarily caused by the formation of intrastrand cross-links in DNA, resulting in apoptosis. Division-arrested cells have been used for years as feeder layers for the culture of embryonic stem cells. Thomas Livelli and colleagues from Cellular & Molecular Technologies (CMT), now part of InVitrogen (http://www.invitrogen.com), have developed protocols to make the process less severe [36]. They showed that pulse treatment (e.g. 1.5 hours) with low doses of mitomycin C resulted in a significant decrease in cell growth without toxicity [33]. Signal transduction in cells treated with mitomycin C was maintained. The division-arrested cells were applied successfully in second messenger assays for GPCRs (i.e. calcium and cAMP), and luciferase and β-lactamase reporter gene assays [33,34] (Table 1). The pharmacology of reference compounds was compared in division-arrested cells and dividing cells that had continuously been maintained in culture. In several instances mitomycin C was found to influence the pharmacology of compounds, resulting in either a decrease [32] or an increase [33] in their potency. As these shifts remained within one log unit, one may reason that the use of division-arrested cells will not significantly affect the overall hit rate and thus the chance of success of a HTS campaign. Economical arguments may further favour the use of division-arrested cells, as it allows cell line propagation for HTS to be outsourced.

Division-arrested cells have also been used successfully for a high-content assay measuring the cytoplasmic—nuclear translocation of the transcription factor NFκB [37]. Division-arrested cells actually gave better results in cellular imaging than non-treated cells. Mitomycin C treatment slightly increased cell and nuclear volume, resulting in more pixels to quantify, contributing to improved image resolution.

Frozen cells

In lead optimization projects and structure–activity relationship (SAR) analyses, deviations of the measured potency or efficacy

TABLE 1

Literature examples of the use of cryopreserved and division-arrested cells

Target name	Target class	Host cell line	Cell supply	Assay readout	Ref
Very late antigen-4 (VLA-4)	Adhesion molecule	Ramos	Frozen	Fluorescence	[30]
Hepatitis C virus replication	Replication proteins	Huh-7	Frozen	β-lactamase reporter	[31]
Epidermal growth factor receptor	Protein kinase	A431	Division-arrested	Receptor phosphorylation	[32]
MIP3α promoter reporter	Pathway	HEK293	Division-arrested	Alkaline phosphatase reporter	[32]
NFκB pathway reporter	Pathway	HEK293	Division-arrested	Luciferase reporter	[33]
5HT2c receptor	GPCR	NIH3T3	Division-arrested	Ca^{2+} (FlexStation)	[33]
Gαq-coupled receptor[a]	GPCR	CHO	Frozen and divison-arrested	Ca^{2+} (FLIPR) β-lactamase reporter	[34]
Gαs-coupled receptor[a]	GPCR	CHO	Frozen and division-arrested	Enzyme fragment complementation	[34]
Potassium channel[a]	Ion channel	HEK293	Frozen and division-arrested	Ca^{2+} (FLIPR)	[34]
NFκB translocation	Transcription factor	A549 HeLa	Division-arrested Division-arrested	High content High content	[37]
hERG	Ion channel	CHO	Frozen	Rb^{+} efflux	[38]
Pregnane X receptor	Nuclear receptor	HepG2	Frozen transients	Luciferase reporter	[39]

[a] Target name was not disclosed in the article.

BOX 1

Advantages of the use of cryopreserved cells for cell-based screening

- Improved consistency of cell-based assay results. Once frozen, the same cell batch can be used over a long period of time.
- Increased flexibility. New assays can start at any moment when compounds arrive for testing.
- Reduced costs. Time spent to maintain cell lines in culture in parallel to drug screening activities is saved. Consequently, the use of cell culture reagents, disposables and cell culture facilities is reduced.

value from the real value would generally not be acceptable. Lead optimization studies usually run over a long period of time, from several months up to a few years. Consistency of cellular responses during the whole course of the project is essential.

Cell lines are usually subcultured twice a week and scaled up for each assay. This subculturing and upscaling is usually repeated in cycles over a period of several months. The use of cryopreserved cells, grown in a large single batch and stored in the freezer, provides significant advantages (Box 1). In contrast to the division-arrest technology, cryopreservation *per se* has generally no effect on the pharmacology of compounds and can be applied to many cell types and assays [30,31,34,38,39] (Table 1). The examples described in literature include cell types that are widely used in drug screening, such as CHO and HEK293, and readouts such as β-lactamase and FLIPR [34].

The procedures for freezing and resuscitation of cells should be optimized for each cell line, but the standard mixture for cryopreservation of cells consisting of 10% dimethylsulfoximide/10% serum works generally well. Thawing of the ampoule with cells just before the assay works well for most cell lines. However, some cell lines require culturing for 2–3 days to obtain the best results.

Figure 1 shows a comparison of luciferase reporter gene assays with 'frozen' cells and with cells that have continuously been maintained in culture ('cultured cells'). In all assays *Z'*-factors were >0.5 with robust *s*/*n*. The potencies (EC_{50}) of reference compounds, 32 low molecular weight agonists and antagonists in total, were compared. The average of the ΔpEC_{50} values was 0.0006, which means that there was no shift in sensitivity when 'frozen' cells instead of 'cultured' cells were used. Similar results were obtained for assays with other GPCRs and other readouts (including cAMP, calcium and β-lactamase).

Louise Stjernborg and colleagues from AstraZeneca (Mölndal, Sweden) described the use of frozen cells for the screening of compound side effects on human *ether-a-go-go* ERG (hERG) channel with a rubidium efflux assay [38]. In their organization, hERG channel screening was not done every day but on a monthly basis, when sufficient new compounds were assembled. The use of frozen cells resulted in a significant saving of time spent on cell culture maintenance.

Recently, a combination of the use of cryopreservation and transient transfection of cells was described [39]. Cryopreserved and freshly transfected HepG2 cells performed equally well in a luciferase reporter gene assay for the pregnane X receptor (PXR) [39]. However, cryopreserved cells demonstrated less inter-assay variation. A good correlation ($r^2 > 0.95$) of drug potencies and efficacies was found between cryopreserved and freshly transfected cells [39].

In summary, the use of frozen cells in drug discovery has three advantages. Firstly, flexibility is increased, because new assays can start at any moment. Secondly, data quality is improved, as all testing results for a certain compound in a certain assay can be generated with the same batch of cells. Thirdly, working with frozen cells substantially reduces the time spent on cell culture work, in particular the maintenance of cell lines, and consequently the use of cell culture facilities, materials and disposables.

Outlook

Cell-based functional assays are becoming an increasingly important tool for drug discovery. Simultaneously, there is a continuous development of cell-based technologies and approaches to make HTS more robust and less sensitive to false positives. This may be achieved, for example by combining two different readouts for the same signal transduction cascade in one assay by multiplexing [40]. Functional cell-based assays allow drug discovery on targets that could not be addressed in biochemical screens. One example is the identification of inhibitors of inducible nitric oxide synthase (iNOS) that have been discovered with a cell-based screen and that prevent dimerization of the enzyme [41]. Pathway screens can address protein classes and cellular mechanisms that previously have not been considered, for instance protein translocation. Inhibitors of the translocation of the transcription factor forkhead (FKHR) from the cytoplasm to the nucleus have been identified in cell-based screens with GFP fusions [42,43]. Also, for these innovative cell-based approaches cryopreserved cells may be explored.

Cell lines used for HTS are usually derived from immortalized human cell lines (e.g. HEK293) or rodent cell lines (e.g. CHO) that have been manipulated by recombinant DNA technology to express the target of interest. Cultured primary cells, platelets or neurons possess characteristics that often better represent *in vivo* physiology than immortalized cell lines. Assays in these cell types could give a better prediction of the activity of compounds in *in vivo* models and in the clinic, and thus may contribute to a decrease in attrition in the whole process of small molecule drug discovery. Primary cell types, platelets and neurons are, however, in short supply and are therefore rarely used for primary drug screening. However, the use of cryopreservation techniques could facilitate this.

The use of cryopreserved cells and/or division-arrested cells can provide immediate cost savings in lead discovery and optimization but is also applicable in phases of the drug discovery process upstream and downstream, such as target validation and ADME/Tox. Cryopreserved hepatocytes derived from multiple species, including rat, dog and monkey, are used to address drug metabolism [44]. Mitomycin-treated cell batches for screening can be purchased from commercial suppliers. As these cells cannot grow, the cell batches are treated as disposables. A single assay is therefore much cheaper than when a stable reporter cell line needs to be purchased. The generation of stable cell lines is laborious and for a number of targets has proven to be very difficult, presumably because of detrimental effects of target expression on cell growth. Novel transfection reagents yield transfection efficiencies that are high enough to perform functional assays with transiently transfected cell populations. These transiently transfected pools can be prepared in large batches and cryopreserved.

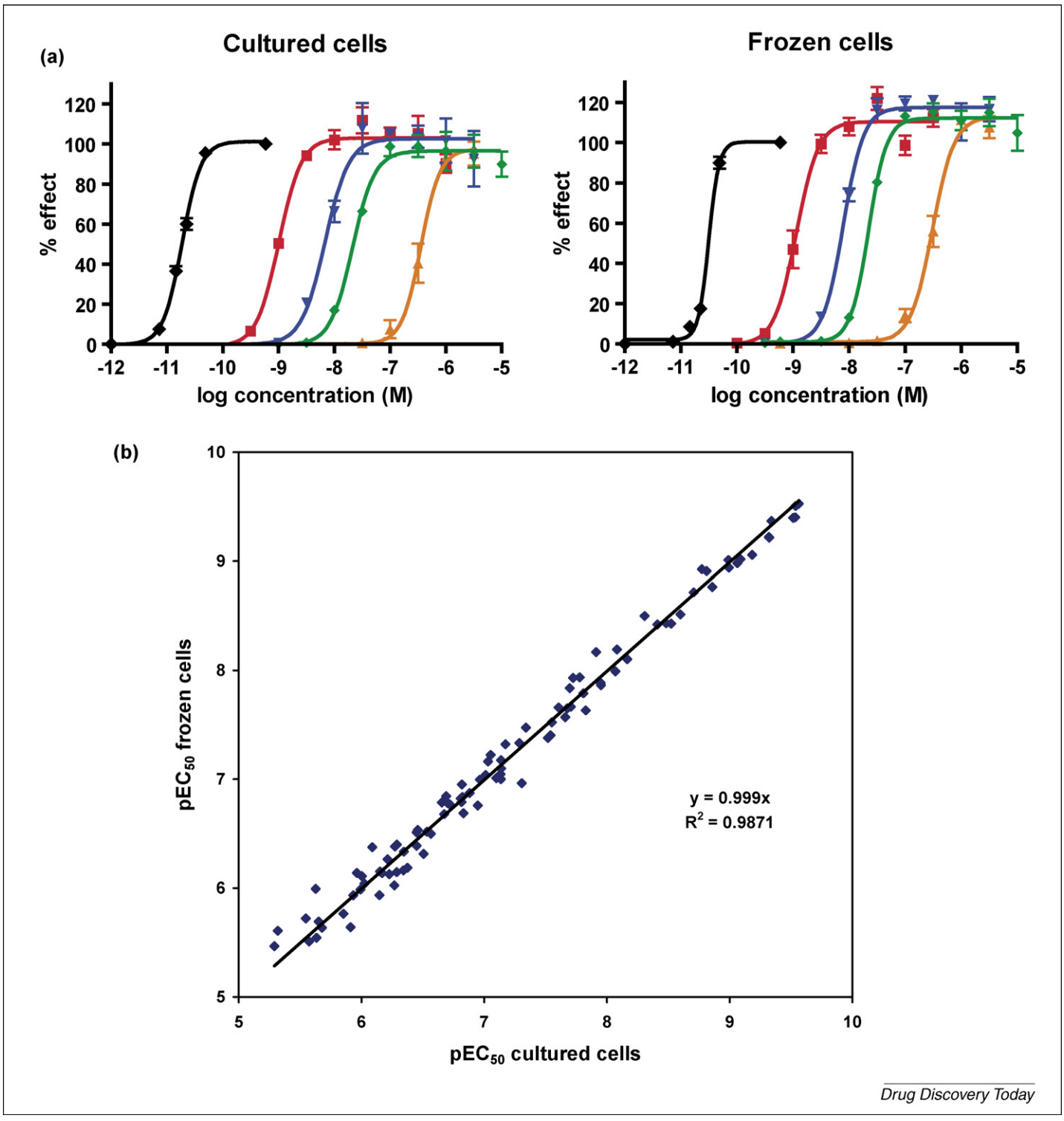

FIGURE 1

Comparison of 'cultured' and 'frozen' cells in lead optimization. (a) Dose–response curves of various FSH receptor agonists in luciferase reporter gene assays with 'cultured' or 'frozen' cells. The black curve is the natural glycoprotein ligand (FSH). The red, blue, green and orange curves are four synthetic low molecular weight (LMW) agonists. Luciferase activity was measured by chemoluminescence using LucLite™ (Perkin–Elmer), after incubation of the cells for four hours with compound [26]. (b) Comparison of the potency of LMW FSH receptor agonists in assays with 'cultured' or 'frozen' cells. One hundred fifteen pEC_{50} of LMW compounds generated with cultured cells were plotted against pEC_{50} values obtained with frozen cells. Data are based on 24 luciferase reporter gene assays, each containing 32 LMW compounds.

Conclusion

Recent advances in detection technologies have enabled an increased use of cell-based functional assays for GPCR drug discovery. Screening assays that use live cells and imaging-based high-content assays improve the quality of HTS and decrease the time needed to select the true hits and to discard technology artefacts. When working with cell-based assays, good logistics and scheduling are a prerequisite. The use of cryopreserved cells removes some of

this burden, because it allows separation of cell culturing from drug-screening activities. The use of cryopreserved and division-arrested cells has been validated for the most commonly used cell lines and assays in drug screening. Cells can now be used as reagents, just like enzymes in biochemical assays. The use of cryopreserved cells increases the overall flexibility of cell-based drug discovery. Consistency of assay results improves data quality. Time and resources can be saved without the need of any infrastructural investment.

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