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Cell-based assays

SPECIAL

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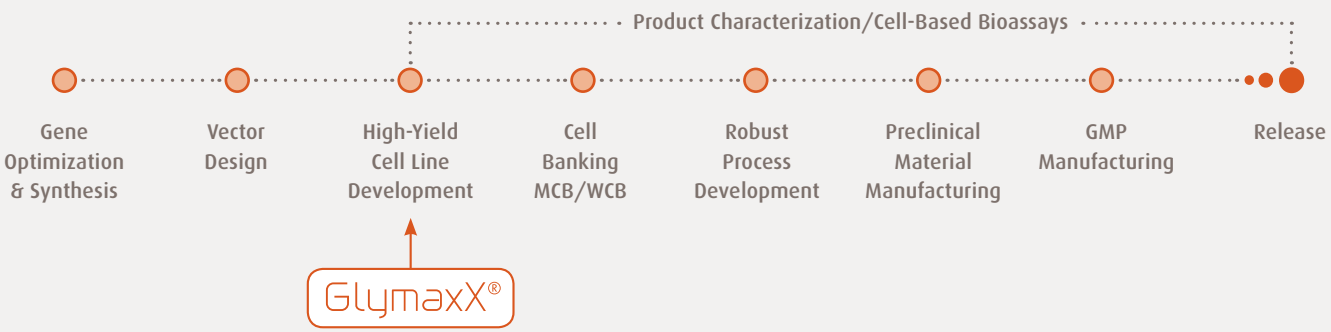
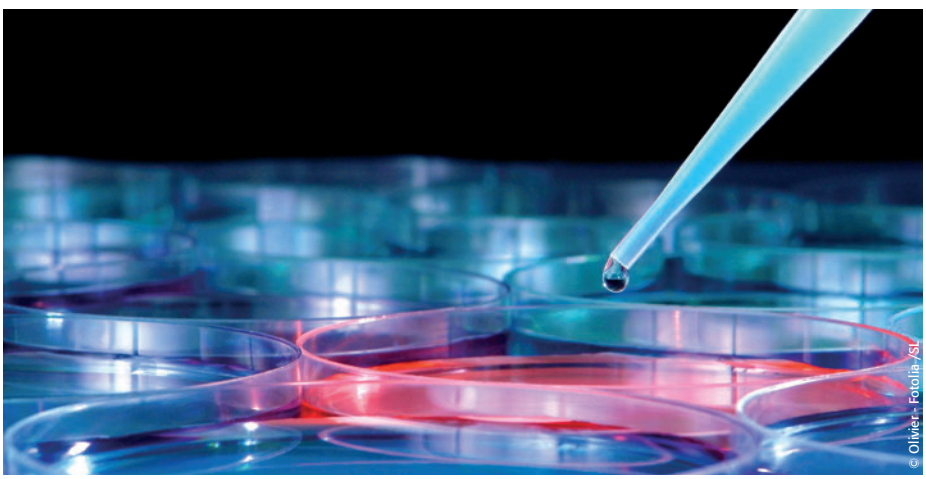


INTRO

The world in a dish

The verdict is still out. Will the classic approach to identifying new drugs – phenotypic screening – prevail over its younger sibling, target-based drug discovery ... also known as "reverse pharmacology"? The former looks at the effects, or phenotypes, that compounds cause in cells, tissues or organisms, while the latter records the effects of compounds on a purified target protein via in vitro assays. Both have drawbacks. Even if there's a desired phenotypic change, it's almost impossible to identify the relevant molecular target. And even if a purified target is altered in a desired manner, that's no guarantee it will have the desired effect in an intact biological system. In the last few years, technological advances such as high-content screening (HCS) in 3D cell cultures have tipped the balance in favour of phenotypic screening. The effects of drugs tested on these complex cell structures are thought to resemble the in vivo setting far better than screening on a 2D monolayer ever could.



Will 2D monolayer cultures soon be a thing of the past?

With breakthroughs in cancer or Alzheimer's therapy taking their time in coming, the industry is keeping a sharp lookout for silver bullets. A 2010 Markets And Markets study of the global top 10 drug discovery technologies – which included high-throughput screening, bioanalytical instruments and pharmacogenomics – predicted annual growth of almost 12% over five years, estimating the market would reach US$54bn by 2015. High-throughput screening accounts for about a third of that growth in the prediction. Along with drug discovery approaches, toxicity testing and compound profiling of candidates in the early clinical stage should also contribute to growth in the sector. Focusing on the fraction of the high-throughput screening market related to cell imaging and analysis systems, Market And Markets foresaw a market volume of US$362.2m in 2013. That number is expected to reach US$615.8m by 2018, growing at a CAGR of 11.2%. In the long run, systems that are more accurate, easier to use and that have a higher throughput rate will prevail. Trends to watch include induced pluripotent stem cell (iPSC) technology (see p. 34) and label-free technologies. These increasingly robust, highly sensitive, automatable tools for measuring live, native cells in real time don't require dyes, engineered cells, tags or special reagents.

Venture capitalists are also acknowledging the growing demand for HCS products. For instance, in November 2011, the Entrepreneurs Fund (UK) and the mutual insurance company Sham (France) led a €7m Series C round for Cytoo SA, a French company that offers cell products and services centered around their patent-protected micropatterning technology. And in late 2011, Kernel Capital invested €0.8m in Biocroi Ltd., an Irish company that develops multi-well microplates for HCS. But Europe aside, analysts say most of the growth in the HCS market is expected to happen in China, Japan, Singapore and India. That's why moves like the June 2012 collaboration between GE Healthcare (UK) and BGI (China) to conduct research on stem cell-derived assays for drug discovery and toxicity testing applications deserve special attention.

Competing models

The search for more biological relevance has propelled different technologies. Cytoo dubs its system 2D+ culture. Real 3D culturing methods include hanging drop spheroids and 3D co-culture platforms. Their biggest plus is that the nutrient, drug, and oxygen mass-transfer gradients established in these self-assembled cellular microenvironments produce drug response profiles that more closely resemble in vivo responses. Their cell formations also possess physiological cell-to-cell contacts, and secrete their own extracellular matrix – which is especially useful in the context of in vitro tumour modeling.

In summary, new culturing approaches try to recreate the mechanical and chemical gradients that cells are exposed to in the body – those which regulate pathways controlling proliferation, polarity, and differentiation. With screening methods getting better and better, the biggest advantage of 2D monolayer cell cultures – convenience – will likely topple soon. The ability to identify a causative molecular target for a particular substance has also improved steadily in the past few years, and computational approaches can already identify putative targets more rapidly and less expensively than current experimental approaches.

DRUG DEVELOPMENT

Antibody testing for Fc receptor binding

Christian Demmler, Michael Sacharjat and Christoph Giese, ProBioGen AG

Binding and cell-based bioactivity assays are used extensively in drug discovery and drug development for candidate identification and selection. Only a reduced set of analytical methods, however, is applied to demonstrate and confirm the quality of the drug product, for comparability exercises in the analysis of in-process controls (IPCs) and later in potency tests for batch release.

Triggered by the new regulatory requirements from December 2012 for biosimilar antibodies in the EMA region[1], the need for extended characterisation of biosimilar antibody products in the late preclinical stage has become an imperative. Analogous regulations are expected from the FDA. Biosimilarity to an originator product now has to be demonstrated by a defined panel of assays, irrespective of the intended therapeutic relevant mode of action (MoA). In the past, biosimilarity was assessed in an individual decisionmaking process under scientific advice on a case-by-case basis. But similarity assessment now covers all antibody-relevant properties: Fab-associated functions (binding, neutralisation, receptor activation, or blocking), Fc-associated functions (effector functions) and complement activation. There is now heavy demand for the following broad panel of assays to test cell-based bioactivity and binding:

Cell-based bioactivity:
- Antibody-dependent cellular cytotoxicity (ADCC)
- Complement-dependent cytotoxicity (CDC)
- Antibody-dependent cellular phagocytosis (ADCP)
- Target cell binding

Binding activity (ELISA-based or label-free):
- Ligand binding
- Fc receptor binding properties for the different receptors (FcγRI, FcγRII, FcγRIII, FcRn)
- C1q-binding

Table 1: The Fc receptor family – cellular distribution and different binding preferences and affinities to antibody subtypes

Name	FcγRI	FcγRIIA		FcγRIIB		FcγRIIC	FcγRIIIA		FcγRIIIB	FcRn
CD	CD64	CD32a		CD32b		CD32c	CD16a		CD16b	Not assigned
Function	Activating	Activating		Inhibitory		Activating	Activating		Activating Decoy	IgG recycling and transport
Cell populations (selection)	Macrophages Neutrophils Eosinophils DC	Macrophages Neutrophils Eosinophils		Macrophages Neutrophils Eosinophils B Cells		NK cells Macrophages Neutrophils	NK cells Macrophages		Eosinophils Macrophages Neutrophils FDC Eosinophils Basophils	Monocytes Macrophages DC Epithelial cells Endothelial cells Hepatocytes
Relevant polymorphisms	n.d.	131H	131R	232I	232T	n.d.	158V	158F	NA1/NA2/SH	n.d.
IgG affinity [1/M]										
IgG1	$7x10^7$	$4x10^6$	$4x10^6$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^6$	$1x10^6$	$2x10^5$	$5x10^6$
IgG2	No binding	$8x10^4$	$5x10^5$	$3x10^4$	$3x10^4$	$3x10^4$	$8x10^4$	$3x10^4$	No binding	$5x10^6$
IgG3	$6x10^7$	$1x10^6$	$1x10^6$	$2x10^5$	$2x10^5$	$2x10^5$	$1x10^7$	$8x10^6$	$1x10^6$	$2x10^6$
IgG4	$3x10^7$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$	$3x10^5$	$2x10^5$	No binding	$5x10^6$

Sources: Bruhns et al. Blood 113(16) (2009), Guilliams et al. Nature Reviews Immunology 14, 94–108 (2014), modified

Target cells and effector cells are both necessary to test Fc-associated bioactivity (effector functions). Primary human NK cells, which have to be freshly prepared from PBMCs (peripheral blood mononuclear cells) from whole blood donations, are used for conventional ADCC assays. The NK cells are either specifically isolated through magnetic bead separation or the PBMC pool is used as a whole, but is adjusted to a defined effector-to-target cell ratio (E:T ratio) for NK cells. The preparation of PBMCs and NK cells is challenging, time-consuming and cumbersome. An assay's robustness and reproducibility depends very much on the quality and variability of the donor material used. Recombinant NK cell-lines or T-cell-derived surrogate models, such as reporter gene assays or recombinant effector cells – which have been introduced to overcome the limitations of primary human NK cells – have produced promising results. In addition to potent effector cells, specific target cell lines are required for ADCC and ADCP. Target cell lines, however, are limited when it comes to many of the popular cancer antibody products. For trastuzumab (Herceptin®) for example, testers know that ADCC assay performance is limited by generally-used target cell lines (BT-474 and SK-BR-3).

Only a target cell line that is exposed to human or rabbit complement serum is required for CDC assays. However, CDC performance *in vitro* is also influenced by target cell-line performance and protective mechanisms on target cells such as membrane-bound complement-inhibiting proteins (CIPs)[2]. It has been shown that target cells can be sensitised against trastuzumab-mediated CDC, for example, by the blocking of CIPs. Exposure of CIP-specific antibodies

increases the susceptibility of target cells being killed by antibody-driven CDC.

Binding assays initially designed for the ELISA format are now used for the characterisation of the Fab-associated functions, such as antigen binding, neutralisation and receptor activation or blocking, as well as to estimate Fc-associated effector functions on a binding level. The analysis of binding performance is extended by the adaption of binding assays to new label-free analytical technologies, i.e. surface plasmon resonance (SPR; Biacore™) or bio-layer interferometry (BLI; Octet™) to quantify affinity to target molecule and binding kinetics.

The C1q-binding assay addresses the formation of the initial step of the complement cascade, and serves as a surrogate model for CDC. C1q is a normal component of the C1 factor molecular complex, and possesses multiple recognition sites for the heavy chain of immunoglobulins. The method is a cell-free binding assay alternative for screening purposes, and a preferred method for release testing and comparability exercises. To confirm biosimilarity, both assays (CDC and C1q-binding) are mandatory.

The Fc part of the antibody binds to different Fcγ receptors (FcγR) on the surface of effector cells, thus inducing a cellular immune reaction. A broad range of FcγR have been described, including FcγRIII (CD16), FcγRIIb (CD16b), FcγRII (CD32), FcγRI (CD64), and subtypes. They are found on different immune cells and show different binding preferences for IG subclasses (see Tab. 1).

Here we have tested trastuzumab candidates for FcγRIIIa binding that have been modified by glyco-engineering (GlymaxX® technology) to adjust different levels of antibody fucosylation (Fig. 1A). The resulting modification of the glycostructure enhances their binding to NK cells, and thus the bioactivity in ADCC. As a consequence, the potency of the modified antibodies directed against tumour or infected cells is substantially increased.

Immunoglobulins are also bound to the Fc neonatal receptor (FcRn), which is found for example on endothelial cells. Endothelial cells control the serum half-life of proteins by vesicular uptake and processing. Good FcRn binding prevents endosomal digestion and ensures release into serum again[3-5]. It also results in a prolonged serum half-life, and provides the opportunity to lower the therapeutic dose. The FcR binding assays are usually performed in the ELISA format, but could also be transferred to label-free binding methods such as SPR or BLI. The FcRn was first recovered in antibody trafficking across placental and mucosal barriers during the fetal and neonatal phase. FcRn binding controls the transfer of maternal serum antibodies to the fetus across the placental barrier and breast-milk-borne antibodies to newborns across the mucosal barrier. As shown in Figure 1B, infliximab candidates have been tested for FcRn binding in full-dose response.

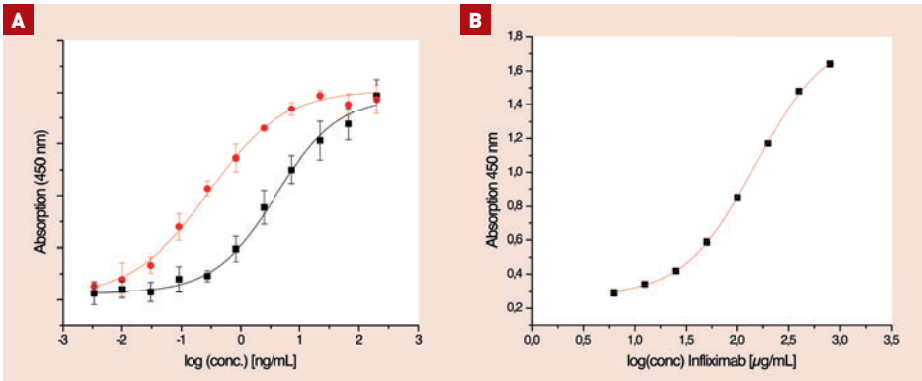


Fig. 1: A. Influence of different levels of fucosylation to FcγRIIIa binding for trastuzumab candidates (ELISA format). Comparison of a GlymaxX® optimized IgG candidate (red cycles) and WT IgG (black squares). B. Dose-response curve for infliximab candidate binding to FcRn (ELISA format).

Bio-layer interferometry (BLI)

ProBioGen uses both the ELISA-based and label-free BLI platform (Octet Red™, Pall) for binding-assays. This provides the opportunity to manage the different binding properties of FcRs, as well as different levels of assay maturation (R&D vs. qualified method) and sample throughput. The BLI technology is an optical-based spectral method for the analysis of dynamic binding kinetics of receptor/ligand or antibody/antigen interactions comparable to SPR. BLI measures biomass deposition on a dip electrode by interference of backscattered coherent light in the 96-well format.

Bioassay Data Analysis

Binding and bioactivity for potency assays are usually derived from full-dose response data using 4-parameter or 5-parameter logistic fit analysis (4P/5P fit). Potency is described by the drug's concentration of half maximum activity (EC_{50}). The maximum induced effect (upper plateau) gives an additional effect, for example, for candidate ranking or comparability testing. Parallel line analysis is applied for the statistical assessment of biosimilarity to the reference drug or the originator's product using commercial software packages (e.g. PLA™, StatLIA™, UNISTAT™, or CombiStats™).

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[1] Committee for Medicinal Products for Human Use (CHMP) guideline: Guideline on similar biological medicinal products containing monoclonal antibodies – non-clinical and clinical issues (EMA/CHMP/BMWP/403543/2010).

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INTERVIEW

One-stop shop for iPS cell data

In an effort to push the country's stem cell field, the UK research financing bodies Wellcome Trust and Medical Research Council in late 2012 forced a £12.75m undertaking to create a catalogue of high-quality adult stem cells, the Human Induced Pluripotent Stem Cells Initiative (HipSci). While the Sanger Institute in Hinxton is in charge of the reprogramming of the received sample cells into iPSCs, and is also performing the genomics and RNAseq analyses, the proteomics work is done in Dundee and the cell phenotyping in London. EuroBiotechNews talked with Davide Danovi, Director HipSci Cell Phenotyping Programme at King's College London, about the prospects iPSCs hold for drug discovery research.

Euro|BioTech|News ?
What is the motivation behind HipSci and how is the project progressing so far?

DANOVI: !
HipSci is born from the intention to create a baseline of detailed biological information from iPSCs of – in the beginning – healthy donors. The number of cell lines we want to establish is similar to that of other initiatives, but our strength lies in the systematic analysis of all the data sets coming from the different platforms. All the information from the different sources is integrated in the end at the EMBL's European Bioinformatics Institute in Hinxton. Soon we will hit the milestone of 100 cell lines. Ultimately, we're aiming for more than 1,000.

Euro|BioTech|News ?
What are you doing exactly at the HipSci Phenotyping Centre?

DANOVI: !
We try to capture the characteristics of each line when exposed to different compounds, among them extracellular matrix proteins, as we are very interested in the factors constituting stem cell niches. After taking images of these lines in each environment, we use high-content analysis to measure the cells' numbers, morphology, arrangement, proliferation and so on. The underlying questions are: Can we capture differences at the cell level within the population of healthy cells? And despite this variability: Can we determine characteristics that distinguish healthy from diseased cells?

Euro|BioTech|News ?
HipSci will run until 2016. What are your plans for the upcoming years?

DANOVI: !
We want to look more carefully into iPSCs as potential models for diseases. In fact, HipSci has just opened a call for proposals in late March where we are seeking samples from patients with rare inherited genetic diseases. We plan that about half of the cell lines will be derived from sick patients.

Euro|BioTech|News ?
So there will be collaborations also beyond basic cell biology research ...

DANOVI: !
Indeed! We intend to involve the clinical genetics community. The collected iPSC data derived from diseased cells and the cell lines themselves will be offered on an open-source basis. We here at King's College will perform assays to see whether a cell that carries a particular disease trait can still respond to a certain condition – and in what way. We have already begun with iPS cell lines from patients affected by ciliopathies. That's a good place to start because the primary cilium is the major cell organelle that senses the environment. So we are exposing these and the healthy cell lines to the same conditions. By discovering differences in their reactions we are "phenotyping" them. The collected data could become an asset for advancing disease modelling and drug discovery. The advantage of iPSCs over other cells is that they are derived from specific patients, and thus represent cells with possibly the highest biological relevance. In fact, the trend is to use cells that are biologically similar to the system studied. Here, when compared to primary cells, iPSCs come in handy. You can expand them much more easily to have the numbers you need for high-content analyses. Besides, when exposing our cell lines to conditions representing different surface chemistries and topologies, we believe that exploring their behaviour in 2D is likely to be more straightforward than exploring complex and not yet validated 3D or co-culture models.

Davide Danovi holds an MD from the University of Milan and a PhD in Molecular Oncology from the European Institute of Oncology. He completed his postdoctoral training working at the University of Cambridge and at University College London, where he developed a screening platform to isolate compounds active on human neural stem cells. He has several years experience in stem cells high-content screening both in academic and commercial settings. Danovi is currently director of the HipSci Cell Phenotyping Programme at King's College London within the Centre for Stem Cells and Regenerative Medicine directed by Fiona Watt.

EUROPE

CDMOs seal the deal

Herleen/New York – In one of the largest transactions in the history of Contract Development and Manufacturing Organisations (CDMO), Patheon and DSM Pharmaceutical Products have completed their merger to form the private company DPx Holdings B.V.

In mid-March, Patheon announced it had finalised its merger with Royal DSM's Pharmaceutical Products business (DSM Pharmaceutical Products) for an estimated US$2.6bn or roughly €1.9bn. The deal resulted in the privately-held DPx Holdings, B.V., which is owned 51% by JLL Partners, a leading middle-market private equity firm, and 49% by Royal DSM, the global Life Sciences and Materials Sciences company.

DPx Holding will have locations all over the world

The new company has emerged as a leading global CDMO for the pharmaceutical and related industries. Led by Jim Mullen, the current CEO of DPx Holdings B.V. and former CEO of Patheon, the new company has its headquarters in Durham (NC, US) and a global footprint that includes 22 locations across North America, Europe, Latin America and Australia with more than 8,000 employees.

"We believe that combining the capabilities and experience of both Patheon and DPP immediately positions us as the pharmaceutical industry's partner of choice," said Jim Mullen, CEO of DPx Holdings B.V. "We are better positioned to add scale, new value-chain capabilities and technologies, as well as expand our end-to-end service offerings to our customers as a global, comprehensive solution provider to the industry."

DPx Holdings B.V. is the corporate parent of a group of business units that is comprised of three distinct brands focused on pharmaceutical services, fine chemicals and products and proprietary technologies. The company's business unit structure is broken down into three existing, trusted brands in the marketplace.

- Pharmaceutical services will operate under the Patheon brand name, and includes the commercial manufacturing capabilities, pharmaceutical product development services (PDS), and the Biologic and Biosolutions offerings.
- Fine chemicals and the active pharmaceutical ingredients businesses will operate under the brand name DSM Fine Chemicals.
- Proprietary products and technologies will operate under the Banner Life Sciences brand name.

The integration of the Biologic and Biosolutions business into the Patheon offering is complementary and a great combination to build an integrated offering for the biomanufacturing sector.

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