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In Vivo Veritas?

Cell-Based Assays for Identifying RTK Inhibitors

By Tea Gunde, Scientist at ESBATech AG

Tea Gunde received her first degree in Biology from the University of Zurich. She then joined ESBATech for her PhD studies on the subject of functional screening systems in yeast, where she was instrumental in the development of the assays for the receptor tyrosine kinases. Her experience and expertise gained during her PhD studies was put to good use in the subsequent exploitation for high-throughput small compound screens for kinase inhibitors and their characterisation in follow-up experiments.

Receptor tyrosine kinases (RTKs) play an important role in many fundamental cellular processes including cell metabolism and survival, as well as cell proliferation and differentiation. Deregulated RTK signalling is critically involved in the development and progression of human cancer. Therefore, RTKs represent attractive targets for anti-cancer therapy. Many methods have been explored to measure the activation and inhibition of RTKs *in vitro* and in living cells. This article discusses the application of functional cell-based screenings for the identification of specific RTK inhibitors.

One of the fundamental mechanisms for cell-to-cell communication in multicellular organisms is the activation of receptor tyrosine kinases (RTKs) – cell surface transmembrane proteins by extracellular ligands such as growth factors. RTKs transduce the extracellular signal, generated by ligand binding, to the cytoplasm via activation of numerous signalling pathways that control processes such as cell growth, differentiation and apoptosis.

Deregulation of these well-balanced pathways contributes to the generation of various human diseases. Enhanced RTK activity is associated with proliferative diseases such as cancer, psoriasis and atherosclerosis, while decreased function of the insulin RTK is associated with various types of diabetes. In addition to their role in tumour growth, RTKs are also implicated in tumour metastasis and angiogenesis. Therefore, RTKs have emerged as key regulators of all aspects of neoplasia, including proliferation, invasion, angiogenesis and metastasis (1,2).

Met, the receptor for hepatocyte growth factor (HGF), regulates cell proliferation and survival, cell migration and invasion, and accounts for the onset and maintenance of normal organ complexity and architecture (3). Under pathological conditions, deregulated Met activity is responsible for cancer invasion, progression and metastasis in transformed cells. This explains why inappropriate expression of HGF and/or Met is found in all types of solid tumours and is often considered as a biological marker for the metastatic potential of a tumour (4). Receptor protein tyrosine kinases for vascular endothelial growth factor (VEGFR-1 and VEGFR-2) are key regulators of angiogenesis, the process by which new vasculature is developed (5). Tumour cells have an absolute requirement for a persistent supply of new blood vessels that provide the tumour with oxygen and nutrients and that facilitate metastasis. Therefore, induction of angiogenesis through VEGFRs is essential for progressive tumour growth. More recently, Eph receptors, first shown to be important regulators of axon path-finding and neuronal cell migration, have been associated with vascular growth and remodelling as well.

Given the importance of RTKs in human disease, potent and selective tyrosine kinase inhibitors represent a new and important class of drug candidates (1,2). The clinical value of small molecule RTK inhibitors is now firmly established with

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the success of Gleevec (STI-571 or imatinib mesylate), an inhibitor of the cytosolic tyrosine kinase bcr-abl and the receptor tyrosine kinases c-kit and plateled derived growth factor receptor (PDGFR). Gleevec is used for the treatment of chronic myeloid leukemia, gastrointestinal tumours and metastatic dermatofibrosarcoma protuberans, diseases that are caused by constitutive activation of bcr-abl, c-kit and PDGFRs, respectively (6). Iressa, a potent EGFR and Her-2 kinase inhibitor, is marketed for the treatment of nonsmall cell lung carcinoma (7). In order to prevent tumour angiogenesis, considerable efforts have been undertaken to screen for VEGFR antagonists. However, early clinical trials with several small molecules targeting the VEGFR kinase, for example SU5416, were disappointing and the search for more potent inhibitors of this RTK continues.

CELL-BASED VERSUS BIOCHEMICAL ASSAYS FOR RTK INHIBITOR SCREENING

Traditionally, kinase activity has been measured *in vitro* with radioactivity-, colorimetric- or fluorescence-based enzyme assays using the isolated kinase and an exogenous peptide or protein substrate. The first cell-based assay that was adapted to a multi-well format measured ligand-mediated EGFR receptor phosphorylation using a traditional ELISA (8). In the last few years, cell-based assays have become an increasingly attractive alternative to *in vitro* biochemical assays for HTS. Functional cell-based assays provide a number of advantages over *in vitro* assays. First, *in vivo* analysis of kinase activity does not require purification and characterisation of large quantities of active enzyme. Moreover, cell-based assays monitor the activity of a kinase towards its endogenous targets in the cellular environment, whereas the substrates used for *in vitro* kinase assays may not be optimal for some kinases. Secondly, within cells the activity of an RTK is regulated under physiological conditions. Thirdly, cellular assays can immediately select against compounds that are generally cytotoxic and can provide information on cell permeability, stability and solubility of the inhibitor.

Most protein kinase inhibitors identified by biochemical screens with isolated targets are ATP competitive because, in the *in vitro* assay, they are selected to inhibit the active form of a kinase. Therefore, the development of compounds with sufficient potency to compete with the high intracellular ATP concentrations can be difficult. Moreover, due to the structural similarity of the catalytic domains of kinases in general, the selection of highly specific inhibitors also represents a problem. Some inhibitors, however, might exert their effects by suppressing activation of a kinase rather than by blocking its activity. Crystal structures of the complex between Gleevec and abl showed that Gleevec inhibits the bcr-abl kinase by binding with high specificity to the inactive conformation of the kinase, thus preventing its activation (9). Since tyrosine kinases are quite similar to one another in their active state, but are structurally distinct in their inactive state, Gleevec's 'conformational specificity' contributes to the high selectivity of this compound. When assayed *in vitro*, Gleevec is a much more potent inhibitor of inactive unphosphorylated abl than of active phosphorylated abl. In intact cells, however, effective inhibition of the constitutively active bcr-abl kinase occurs because the susceptible, inactive kinase conformation is regenerated by the action of protein tyrosine phosphatases, thus maintaining an equilibrium between the two forms. In addition, Gleevec probably binds to newly synthesised bcr-abl, thereby preventing autophosphorylation and activation of the kinase. Therefore, cell-based assays allow identification of inhibitors that can function either by binding to kinases in their active conformations or by blocking their inactive state.

CELL-BASED ASSAYS TO MEASURE RTK ACTIVITY

Reporter Gene Assays

Reporter gene assays are configured to detect downstream transcriptional consequences of RTK activation and inhibition. These assays are based on the use of cell lines that are engineered to contain a reporter gene such as luciferase, galactosidase or green fluorescent protein (GFP) under the control of a pathway-specific promoter. For example, in order to determine insulin RTK activity in whole cells, investigators have established a reporter gene assay that is based on STAT 5-dependent luciferase gene expression (10). Reporter gene expression can be induced by insulin because the activated insulin receptor phosphorylates and activates the transcription factor STAT 5. Addition of the insulin RTK inhibitor AG1024 down-regulates luciferase expression in a dose-dependent manner.

Generally, transcriptional readouts are simple to detect and can monitor, fairly specifically, upstream signalling events if the promoter controlling expression of the reporter gene is well

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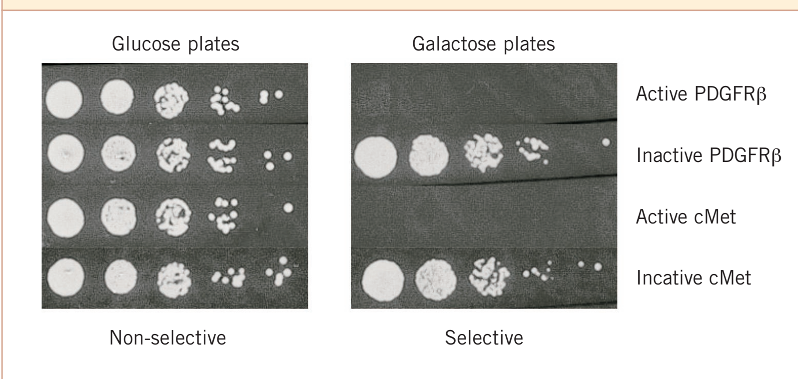
matched to the upstream effector RTK. In addition, reporter enzymes provide a highly amplified signal. However, reporter gene assays are subject to non-specific interference by compounds that do not act directly on the RTK, but target intermediary signalling proteins of the given pathway.

FRET Assays

Fluorescence resonance energy transfer (FRET) occurs when two fluorophores with overlapping donor emission and acceptor excitation spectra are brought in close proximity. Excitation energy is transferred from the donor to the acceptor and results in a decrease in donor intensity and a concominant increase in acceptor intensity. In intermolecular FRET, the donor and acceptor fluorophores reside on different molecules, whereas in intramolecular FRET, the two fluorophores reside on the same molecule.

Figure 1: Growth of Yeast Cells upon Inactivation of Protein Kinase Activity

Inducible expression of constitutively active PDGFRβ and cMet causes a kinase-dependent growth inhibition of yeast cells. Inactivation of the kinase activity through mutation of a conserved residue in the ATP binding site suppresses the growth inhibitory effect. Equal amounts of serially diluted yeast cultures were spotted onto glucose plates, on which expression of the RTK genes is repressed and onto galactose plates, on which RTK gene expression is induced.



Bastiaens and co-workers used intermolecular FRET to study autophosphorylation dynamics of the EGFR in single cells (11). EGFR phosphorylation was monitored using a GFP-tagged EGFR and a microinjected Cy3-conjugated anti-phosphotyrosine antibody. EGFR activation resulted in binding of the anti-phosphotyrosine antibody to phosphorylated tyrosine residues in the EGFR C-terminal tail, which brings the acceptor and donor fluorophore into close proximity leading to FRET.

More recently, intramolecular FRET has been applied to detect activity of several kinases including abl, EGFR and the insulin receptor in living cells (12,13). FRET-based indicators consist of fusions of cyan fluorescent protein (CFP); a phosphotyrosine binding domain, a substrate for the relevant kinase; and yellow fluorescent protein (YFP). Phosphorylation of the indicator, and subsequent binding of the phosphotyrosine binding domain to the phosphorylated substrate domain, results in a conformational change inducing FRET between the two fluorescent units.

A major drawback for the use of intermolecular FRET assays in HTS is the need to microinject the antibody into cells. Intramolecular FRET is complicated by the fact that most kinases possess overlapping substrate specificities and therefore FRET-based indicators often lack specificity for a given RTK. In addition, FRET changes are often limited by non-optimal fluorophore orientations or spacing.

A Yeast-Based Assay for the Identification of Modulators of RTK Activity

Mammalian cell-based assays can be expensive and time-consuming. Moreover, due to the complexity of signal transduction and cellular function in mammalian cells, measurement of a specific effect on a given target can be difficult. Yeast genetic systems have emerged as powerful tools for measuring the activity of defined human proteins (14). Therefore the yeast *Saccharomyces cerevisiae* represents an inexpensive and rapid alternative for the analysis of RTKs in a heterologous, yet cellular, eukaryotic environment.

Researchers have developed a cellular growth selection system in yeast to identify specific inhibitors of human RTKs. In this system, yeast cells that express constitutively activated human RTKs can grow only upon inactivation of the protein kinase activity (see Figure 1). The positive readout for RTK inhibition avoids selection of toxic compounds because they inhibit cell proliferation *per se*. This reduces the number of false positives that would show up in an assay with a negative readout.

The fact that yeast does not have any endogenous RTKs and no *bona fide* tyrosine kinases, offers the advantage of a null background for the expression of mammalian RTKs – a privileged situation that could not be obtained in mammalian cells. Specificity of inhibitors can be addressed in yeast with a broad panel of human RTKs. Furthermore, endogenous serine/threonine and dual-specificity protein kinases that should not be inhibited if cell growth has to occur, provide a built-in specificity control.

A critical limitation associated with yeast-based analysis of small molecules is imposed by the lower drug sensitivity of wild-type yeast compared with higher eukaryotic cells. This difference in drug sensitivity has been mostly attributed to very efficient compound efflux by yeast multidrug ABC transporters that lower the intracellular concentration of compounds. However, intracellular drug accumulation in yeast can be enhanced by deleting the major ABC transporters (15). We have shown that, in contrast to wild-type yeast, a strain lacking the three most prominent yeast ABC transporters (PDR5, SNQ2 and YOR1) is sensitive to the small molecule tyrosine kinase inhibitor Gleevec. Administration of Gleevec to PDGFRβ expressing yeast cells deleted for the three mentioned ABC transporters was shown to restore growth under selective conditions in a dose-dependent manner (see Figure 2). Growth

Figure 2: Selection for Inhibition of RTK Activity in a Yeast Strain Lacking the Three Major Efflux Pumps

Only PDGFRβ expressing yeast cells lacking the three major ABC transporters PDR5, SNQ2 and YOR1 are sensitive to the PDGFRβ-specific kinase inhibitor Gleevec. Wild-type yeast strains and yeast strains deleted for the three ABC transporters were exposed to increasing concentrations of Gleevec. The level of growth restoration at different concentrations of the compound was determined by OD595 light scattering measurement of the cell cultures. The addition of 100μM Gleevec completely restored growth of PDGFRβ expressing yeast cells.

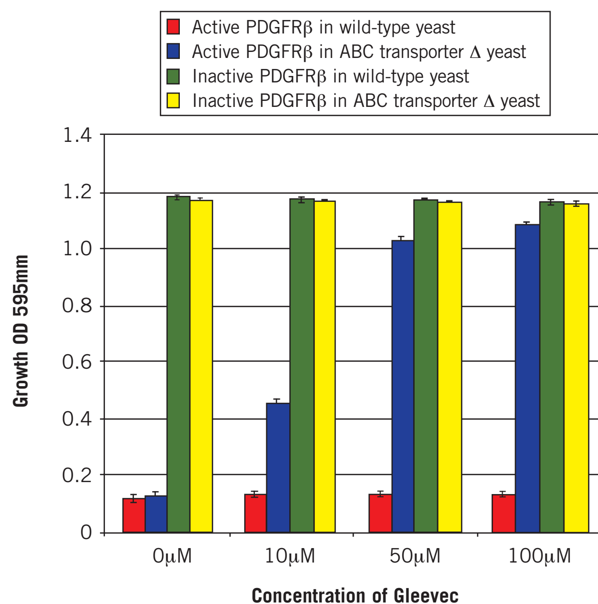


Table 1: Specificity Profile of Gleevec in Yeast Cells Expressing Human RTKs

Human RTK expressed	Growth stimulation by Gleevec
PDGFRβ	+++
PDGFRα	+++
MET	-
RON	-
IGF-1R	-
INSR	-
FGFR-1	-
TRKA	-
EPHB2	-
EPHB4	-
AXL	-
TEK	-
RET	-

inhibition caused by other RTKs, unaffected by Gleevec, was not relieved in the presence of this inhibitor (see Table 1). These results demonstrate that an appropriately engineered yeast-assay can serve as a screening platform for RTK inhibitors, combining the advantages of *in vitro* with those of cell-based assays.

CONCLUSION

There is constant debate as to the benefits of discrete isolated target screening over cell-based screening. Most small molecule RTK antagonists that are currently in clinical trials have been identified with biochemical methods measuring the activity of the isolated kinase domain. However, cellular screens provide promising tools for the identification of noncytotoxic and metabolically stable drug candidates that can permeate cellular membranes. Both biochemical and cell-based approaches are undoubtedly of value for lead discovery, and a combination of these approaches has proven extremely useful for drug discovery. ◆

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