Cell-based kinase assays in HTS

Potential and limitations for primary and secondary screening

Benjamin Bader

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• Introduction
• General considerations cell-based kinase assays
• EFC-assays for Tyr-Kinases
• TR-FRET systems: HTRF and Lanthascreen assays for the PI3K / Akt / mTOR pathway
• Summary & conclusions
## Kinases as drug targets

### Registered kinase inhibitors

<table>
<thead>
<tr>
<th>Compound</th>
<th>Kinase target</th>
<th>Cancer target</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imatinib (Gleevec, STI571)</td>
<td>ABL, PDGFR, KIT</td>
<td>CML, Ph+ B-ALL, MML, CML, GIST</td>
<td>Novartis</td>
</tr>
<tr>
<td>Gefitinib (Iressa, ZD1839)</td>
<td>EGFR</td>
<td>NSCLC</td>
<td>AstraZeneca</td>
</tr>
<tr>
<td>Erlotinib (Tarceva, OSI-276)</td>
<td>EGFR</td>
<td>NSCLC, pancreatic cancer</td>
<td>OSI, Genentech Inc, Roche</td>
</tr>
<tr>
<td>Lapatinib (Tykerb, GW2016)</td>
<td>EGFR, HER2</td>
<td>Breast cancer</td>
<td>GSK SmithKline</td>
</tr>
<tr>
<td>Dabrafenib (Sprycel, BM-35425)</td>
<td>ABL, PDGFR, KIT, SRC</td>
<td>CML</td>
<td>Bristol Myers</td>
</tr>
<tr>
<td>Nilotinib (Tasigna, AMN107)</td>
<td>ABL, PDGFR, KIT</td>
<td>CML</td>
<td>Novartis</td>
</tr>
<tr>
<td>Sunitinib (SutENT, SU11248)</td>
<td>VEGFR-1–3, KIT, PDGFR, RET, CSFIR, FLT3</td>
<td>RCC, GIST</td>
<td>Pfizer</td>
</tr>
<tr>
<td>Sorafenib ( Nexavar, Bray 48-9005)</td>
<td>VEGFR2, PDGFR, KIT, FLT3, BRAF</td>
<td>RCC</td>
<td>Onyx and Bayer Pharmaceuticals</td>
</tr>
<tr>
<td>Pazopanib (Votrient, GF-78934)</td>
<td>VEGFR-1–3, PDGFR, KIT</td>
<td>RCC</td>
<td>GSK SmithKline</td>
</tr>
<tr>
<td>Bosutinib (Afinitor, Rub401)</td>
<td>mTOR</td>
<td>RCC</td>
<td>Novartis</td>
</tr>
<tr>
<td>Tamarotimus (Toriel, CCT-779)</td>
<td>mTOR</td>
<td>RCC</td>
<td>Wyeth</td>
</tr>
</tbody>
</table>

### Recent additions

- Vandetanib: VEGFR, EGFR, RET, thyroid cancer
- Vemurafenib: B-RafV600E, melanoma
- Regorafenib: multiKinase, colorectal cancer
- Citeprotinib: ALK, ROS1, NSCLC
- Bosutinib: BCR/Abl, CLL
- Ruxolitinib: JAK1/2, Myelofibrosis

### Recent advances

- Human kinome: 518 protein kinases + 20 lipid kinases (Manning 2002)
- Currently, ~150 kinase targeted drugs are in clinical development (Fabbro 2012)
- Most registered kinase drugs target Tyrosine kinases, with more Ser/Thr kinase targeted drugs in the pipeline
- Most kinase drugs target the ATP-pocket
- Main indication: oncology
## Kinase Inhibitors in the BAYER Development Pipeline

<table>
<thead>
<tr>
<th>Phase I (11)</th>
<th>Phase II (8)</th>
<th>Phase III (14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer / CDK Inhibitor</td>
<td>Cancer / PI3K Inhibitor</td>
<td>Thyroid Cancer / Sorafenib</td>
</tr>
<tr>
<td>Cancer / Mesothelein-ADC</td>
<td>Cancer / Regorafenib*</td>
<td>Breast Cancer / Sorafenib</td>
</tr>
<tr>
<td>Cancer / PSMA BiTE Antibody</td>
<td>Cancer / MEK-Inhibitor</td>
<td>Adjuvant HCC / Sorafenib</td>
</tr>
<tr>
<td>Cancer / PI3Kα/β Inhibitor</td>
<td>Cancer / Radium-223 Dichloride</td>
<td>Adjuvant RCC / Sorafenib</td>
</tr>
<tr>
<td>Anemia / HIF-PH</td>
<td>Additional Indications / Sorafenib</td>
<td>Major Adverse Cardiac Events / Rivaroxaban</td>
</tr>
<tr>
<td>Heart Failure / Partial Adenosine A1 Agonist</td>
<td>CHF / MR Antagonist</td>
<td>Hemophilia / peg rFVIII**</td>
</tr>
<tr>
<td>Heart Failure / Vaspressin Receptor Antag.</td>
<td>PH / Riociguat (sGC Stimulator)</td>
<td>Hemophilia / rFVIIa**</td>
</tr>
<tr>
<td>Heart Failure / sGC Stimulator</td>
<td></td>
<td>Myopic CNV / Aflibercept</td>
</tr>
<tr>
<td>Bronchiectasis / Neutrophil Elastase Inhibitor</td>
<td>Gram-neg. Pneumonia / Amikacin Inhale</td>
<td>DME / Aflibercept</td>
</tr>
<tr>
<td>Symp. Uterine Fibroids / S-PRAnt</td>
<td></td>
<td>Contraception / LGS 16</td>
</tr>
<tr>
<td>Endometriosis / BAY 1028153</td>
<td></td>
<td>VV Atrophy / Vaginorm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Submental fat removal / ATX-101</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lung Infection / Cipro Inhale</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Skin and Lung Infections / Tedizolid</td>
</tr>
</tbody>
</table>

*Regorafenib is a Bayer compound developed solely by Bayer. In 2011, Bayer entered into an agreement with Onyx Pharmaceuticals, Inc. under which Onyx will receive a royalty on any future global net sales of regorafenib in oncology.

** Combined Phase III

Status February 2013

Selection of major Pharma pipeline projects in clinical Phase I to III
Features of a typical early kinase drug discovery project

1° uHTS
- Biochemical target kinase assay

Retest confirmation
- Biochemical target kinase assay
- Specificity detection control assay

Dose response
- Biochemical target kinase assay
- Off-target assay, selectivity assay
- Mode-of-action assays: competitive ATP, slow binding

Cell-based mechanistic assay
- Downstream substrate phosphorylation or defined cellular phenotype

Cell-based functional assays
- Proliferation, cytokines, migration etc.

Pharmacokinetics

In vivo efficacy

# compounds
- 3 Mio
- 30,000
- 3,000 (HTS-hitlist)
- 300-1000
- 300-1000
- 30-100
- 3

correlation mechanistic – biochemical

• Specific cell-based kinase assays are essential for hit profiling during hit-to-lead phase
• Integration of cell-based kinase assays as early as possible, best case even before hitlist delivery
Key objectives for every assay
(except HCA staining procedures)

- assay-ready plates
- 1536 well format, at least 384 well
- homogenous addition only
- endpoint assays
- frozen cells
- short
- robust
General options for cell-based kinase assays

<table>
<thead>
<tr>
<th>Assay technology</th>
<th>Provider</th>
<th>comments</th>
<th>critical tools</th>
</tr>
</thead>
<tbody>
<tr>
<td>Western Blot</td>
<td>various</td>
<td>heterogenous</td>
<td>phospho-antibody</td>
</tr>
<tr>
<td></td>
<td></td>
<td>low throughput</td>
<td></td>
</tr>
<tr>
<td>Incell Western</td>
<td>various</td>
<td>high content imaging</td>
<td>phospho-antibody</td>
</tr>
<tr>
<td>ELISA-type</td>
<td>various</td>
<td>heterogenous</td>
<td>antibody pair</td>
</tr>
<tr>
<td>Luminex</td>
<td>Millipore</td>
<td>heterogenous</td>
<td>antibody pair</td>
</tr>
<tr>
<td>Surefire / ALPHAscreen</td>
<td>PerkinElmer</td>
<td>homogenous</td>
<td>antibody pair</td>
</tr>
<tr>
<td>HTRF</td>
<td>Cisbio</td>
<td>homogenous</td>
<td>antibody pair</td>
</tr>
<tr>
<td>Lanthascreen</td>
<td>LifeSciences</td>
<td>homogenous</td>
<td>phospho-antibody recombinant cell-line</td>
</tr>
<tr>
<td>EFC - Enzyme Fragment Complementation</td>
<td>DiscoveRx</td>
<td>homogenous</td>
<td>antibody-free recombinant cell-line</td>
</tr>
<tr>
<td>Survival assay</td>
<td>Advanced Cellular Dynamics / Carna</td>
<td>Tyr-kinase activity restores survival of Ba/F3 cells</td>
<td>antibody-free recombinant cell-line</td>
</tr>
<tr>
<td>Pathway reporter assays</td>
<td>various</td>
<td>downstream gene activation</td>
<td>antibody-free recombinant cell-line</td>
</tr>
</tbody>
</table>
EFC-assays for Tyr-Kinases (DiscoveRx)

Technology EFC (enzyme fragment complementation)

EphB4 cellular kinase activity assayed using an enzymatic protein interaction system (Wehrman et al. 2013 ADT)

- Agonist stimulation
- Validation with kinase inhibitors
- Frozen cell and direct assay format

- EFC-kinase technology is suitable for miniaturized frozen cell assays
- limitations: largely restricted to Tyrosine kinases, requires recombinant cells
TR-FRET systems: HTRF and Lanthascreen

**HTRF**
- endogenous phospho-protein
- any cell line expressing protein
- antibody pair required
- HTRF-detection at 620/665 nm

**Lanthascreen**
- overexpressed GFP-phospho-protein
- stable cell line or BacMam transient
- only phospho antibody required
- Lanthascreen detection at 490/520
HTRF and Lanthascreen assays for the PI3K / Akt / mTOR pathway
Introducing the PI3K / Akt / mTOR pathway

- PI3K / Akt pathway is central to cancer formation
- Chemotherapeutic approaches against multiple targets are in the pipelines
Assaying the PI3K / Akt / mTOR pathway

**Lanthascreen study goals:**
- Evaluate BacMam Lanthascreen technology
- GFP-Akt @ two sites (pT308 and pS473)
- GFP-PRAS40 @ two sites (S183 and T246)
- test different cell backgrounds
- validate with reference inhibitors

**HTRF study goals:**
- set up cell-based mTOR kinase activity assay for HTS
- identify optimal cancer cell background
- validate with reference inhibitors

→ validate HTS-compatibility with both formats using a miniaturized 384 well focused screen

(modified from Carlson 2009)
Lanthascreen Assay Development

BacMam technology combined with Lanthascreen Cellular assay

1. Target gene transfer plasmid
2. Recombinant baculovirus DNA
3. Sf9 insect cells
4. BacMam virus

- mammalian cells
- BacMam virus overnight
- GFP-positive cells
- plate cells overnight
- cryopreserve
- thaw & plate cells overnight

(modified from Kost 2007, Carlson 2010)
Lanthascreen – cellular background

**Lanthascreen study goals:**
- Evaluate BacMam Lanthascreen technology
- GFP-Akt @ two sites (pT308 and pS473)
- GFP-PRAS40 @ two sites (S183 and T246)
- test different cell backgrounds
- validate with reference inhibitors

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Type</th>
<th>PI3K Pathway mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC-3</td>
<td>Human prostate cancer</td>
<td>PTEN negative</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Human breast cancer</td>
<td>PI-3-Kinase mutation E545K</td>
</tr>
<tr>
<td>MDA-MB-453</td>
<td></td>
<td>PI-3-Kinase mutation H1047R</td>
</tr>
<tr>
<td>KPL-4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HEK-293</td>
<td>Human embryonal kidney</td>
<td>WT</td>
</tr>
<tr>
<td>U2-OS</td>
<td>Human osteosarcoma</td>
<td>WT</td>
</tr>
</tbody>
</table>

→ MCF7 PRAS40-pT246 selected for further optimization work
Lanthascreen – virus efficiency

- different virus efficiency: PRAS40 with highly efficient expression ($S/B_{\text{max}}$ reached at 1%)
- differences between phosphosites → antibody quality (?)
Lanthascreen – cellular background

- PC3-cells - in contrast to MCF7 - have fully stimulated pathway
  - cave → different pathway mutations have different impact on basal activation
Lanthascreen - optimization

- stimulation time
- stimulation temperature
- antibody concentration
- time cell lysis → read
- DMSO sensitivity
- Volume 10 µl → 5 µl
- frozen cells
Lanthascreen - optimized assay protocol

**Day 1:**
Seed cells into MCF7 cells T-flask (frozen or continuous culture)

**Day 2:**
Transduce MCF7 cells with 1% PRAS40 virus in T-flask

**Day 3:**
Harvest transduced cells and prepare cell suspension in medium + 1% FCS
Dispense 3 µl (5000 cells/well) into assay-ready MTP containing 50 nl cpd.

- 2 h @ 37°C
- Add 1 µl lysis/detection mix
- 2 h @ RT
- Read TR-FRET
MK-2206
allosteric Akt inhibitor

PI-103
PI3K kinase inhibitor

AZD-8055
mTOR kinase inhibitor

- potency of pathway inhibitors depends on incubation time
- Akt and mTOR inhibitors improved IC50 after 2 h
HTRF – assay

**HTRF study goals:**
- set up cell-based mTOR kinase activity assay for HTS using pAkt Ser473 readout
- identify optimal cancer cell background
- validate with reference inhibitors

(modified from Carlson 2009)

Detection

(from Cisbio product insert)
HTRF – cell lines

- MCF7 cells strongest S/B
- frozen cell assay
- suspension cell format
- miniaturized to 1536 well
HTRF - optimized assay protocol

**day 1:**
- Thaw frozen MCF7 cells and prepare cell suspension in medium + 1% FCS
- Dispense 3 µl (4000 cells/well) into assay-ready MTP containing 50 nl cpd.
  - 30 min @ 37°C
  - Add 1 µl lysis buffer
  - 1 h @ RT shake
  - Add 4 µl antibody detection mix
  - 20 h @ RT

**day 2:**
- Read TR-FRET
HTRF – pathway inhibitor validation

IC50 = 79nM

MCF7 cells

PI3K inhibitor PI-103

IC50 = 635nM

AKT inhibitor MK-2206

no effect

IC50 = 163nM

mTORC1 inhibitor Rapamycin

IC50 = 7nM

mTOR inhibitor Ku-0063794

mTOR inhibitor AZD 8055

PI3K inhibitor PI-103

HTRF – pathway inhibitor validation
**Focussed medium throughput screen:**

- BacMAM Lantha PRAS40-pT246
- HTRF Akt-pS473

- 24,300 compounds, kinase targeted library
- 384 well single format
- 69 MTPs
- 5 parallel assay runs (Lantha and HTRF)
Focussed medium throughput screen

- Similar sensitivity of both assays against mTORi
- HTRF: stable and robust assay performance
- Lanthascreen: day-to-day variability in S/B
Focussed medium throughput screen

Distribution neutral controls:

- Neutral controls: similar distribution for both assays

Distribution compounds / donor interference:

- Compounds: strong interference in BacMAM donor channel
Focussed medium throughput screen

<table>
<thead>
<tr>
<th></th>
<th>Lantha</th>
<th>HTRF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interference</td>
<td>high (19%)</td>
<td>low (1%)</td>
</tr>
<tr>
<td>Hit rate</td>
<td>high (1.6%)</td>
<td>low (0.3%)</td>
</tr>
<tr>
<td>Confirmation rate</td>
<td>low (12%)</td>
<td>high (62%)</td>
</tr>
</tbody>
</table>

**Hits > 30% @ 10 µM**
- Lantha Assay: 294 hits, 4686 compounds (19% interference)
- HTRF Assay: 24228 hits, 282 compounds (1% interference)

**Confirmed hits**
- 24 available for retest, 255 compounds (12% confirmation rate)
- 5 available for IC50, 28 compounds (31% confirmation rate)

**IC50 hits**
- 7 available, 25 compounds (31% confirmation rate)
Summary & Conclusions

- Kinases remain interesting target class in pharmaceutical research
- Specific and efficient cell-based kinase assays are essential in pharmaceutical research projects
- Homogenous assay systems fit best to Bayer's lead discovery platform
- Positive experience at Bayer with EFC-technology, HTRF and Lanthascreen
- EFC-technology interesting for Tyr-Kinase uHTS
- Lanthascreen technology positioned for secondary testing
- HTRF positioned for uHTS and secondary testing
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