Developing tests for Bcr-Abl activity and Gleevec resistance in CML patients

A progress report on IMAT R33 CA103235, "Bcr-Abl kinase assays for STI571 sensitivity or response"

Stephen J. Kron M.D.-Ph.D.
The University of Chicago
1973: A chromosome translocation in CML


Cytogenetic testing for molecular diagnosis, monitoring

Janet Rowley M.D.
U. Chicago
Lasker Award 1998
1982: Ph1 chromosome encodes BCR-ABL

Molecular diagnosis and monitoring via unique transcript
Tyrosine kinase enzyme: Active site = druggable target

Owen Witte M.D.-Ph.D.
UCLA
1997: Bcr-Abl kinase blocker kills CML cells and "cures" chronic phase patients

Imatinib mesylate
Gleevec (Novartis)
$2.2B in 2005

STI571

STI571+Abl
J. Kuriyan

Brian Druker M.D.
Oregon Health Sci.
Nobel Prize 200?
2002: Imatinib resistant kinase mutations

1, F317L; 2, T315I; 3, F359; 4, M244; 5, G250; 6, Q252; 7, Y253; 8, E255; 9, M351; 10, E355; 11, V379; 12, L387; 13, H396

C. Sawyers and others
2006: A proliferation of new drugs, but no assays

Cheaper, generic imatinib

Newly approved & on the way

<table>
<thead>
<tr>
<th>Code</th>
<th>Company</th>
<th>Drug</th>
</tr>
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<tbody>
<tr>
<td>AMN107</td>
<td>Novartis</td>
<td>Nilotinib</td>
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<tr>
<td>BMS354825</td>
<td>Bristol-Myers</td>
<td>Dasatinib</td>
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<tr>
<td>CGP76030</td>
<td>Pfizer</td>
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<td>AP23464</td>
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<td>AZD0530</td>
<td>Astra Zeneca</td>
<td>Phase I</td>
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<td>SKI-606</td>
<td>Wyeth-Ayerst</td>
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<tr>
<td>ON012380</td>
<td>Onconova</td>
<td>Phase I</td>
</tr>
<tr>
<td>VX-680</td>
<td>Merck</td>
<td>Phase I</td>
</tr>
</tbody>
</table>

and many more in development…

New clinical challenges

- Rapid testing for Imatinib resistance
- Selection of second-line therapy
- Identifying effective dosage
- Determining failure of STI therapy
Methylcellulose assay for imatinib sensitivity

Semi-solid matrix, supplemented with growth factors, allows individual progenitors to form discrete colonies.

- Cell suspension in MethoCult
- 5 x 10^4 cells/35 mm dish
- 37°C, 14 to 16 d
- Image colonies

<table>
<thead>
<tr>
<th></th>
<th>0 µM</th>
<th>1 µM</th>
<th>10 µM</th>
<th>100 µM</th>
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<tbody>
<tr>
<td>K562</td>
<td><img src="K562_0_%C2%B5M" alt="Image" /></td>
<td><img src="K562_1_%C2%B5M" alt="Image" /></td>
<td><img src="K562_10_%C2%B5M" alt="Image" /></td>
<td><img src="K562_100_%C2%B5M" alt="Image" /></td>
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<td>BaF3/Y253F</td>
<td><img src="BaF3_Y253F_0_%C2%B5M" alt="Image" /></td>
<td><img src="BaF3_Y253F_1_%C2%B5M" alt="Image" /></td>
<td><img src="BaF3_Y253F_10_%C2%B5M" alt="Image" /></td>
<td><img src="BaF3_Y253F_100_%C2%B5M" alt="Image" /></td>
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<tr>
<td>BaF3/T315I</td>
<td><img src="BaF3_T315I_0_%C2%B5M" alt="Image" /></td>
<td><img src="BaF3_T315I_1_%C2%B5M" alt="Image" /></td>
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<td><img src="BaF3_T315I_100_%C2%B5M" alt="Image" /></td>
</tr>
</tbody>
</table>

D. Sher
Technology challenge: Measure Bcr-Abl activity

Criteria for a useful kinase assay
- Detect Bcr-Abl activity in whole cell lysate
- Dynamic range to determine Ki for inhibitors
- Rapid, robust and simple assay, amenable to clinical lab
- Adaptable to high throughput for screening, drug discovery

Abtlide

Abl, BCR-ABL

EIYAAPFAAKKK + ATP $\rightarrow$ EIpYAAPFAAKKK + ADP

Detect:
- ADP
- Phosphotyrosine
- Phosphopeptide

Ignore:
- Cell lysate
- Other kinases
- Phosphatases

Solid-phase assays

Beads versus Chips
Proof-of-principle
Bead-based assay of Bcr-Abl in cell lysates

Glutathione Agarose Bead

GST CrkL domains

ATP
Cell Lysate

Y

+/- Imatinib

Y

Y

P

Glutathione

Anti-phosphotyrosine Western blot
High affinity substrates via Abl binding domain

![Diagram of protein interactions]

<table>
<thead>
<tr>
<th>Protein</th>
<th>GST-Abltide</th>
<th>GST-Abl SH3L-Abltide</th>
<th>GST-Crkl SH3n-Abltide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
</tr>
</tbody>
</table>

Reaction on beads with c-Abl

100 µM Imatinib

<table>
<thead>
<tr>
<th>GST-Abltide</th>
<th>GST-Abl SH3L-Abltide</th>
<th>GST-Crkl SH3n-Abltide</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
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α-p-Tyr

Memcode

D. Wu et al.
Bcr-Abl inhibition assay in K562 and CML cells

**Western blot -- K562 cell steady-state phosphorylation**

<table>
<thead>
<tr>
<th>µM IM pretreatment</th>
<th>0</th>
<th>0</th>
<th>1</th>
<th>1</th>
<th>10</th>
<th>10</th>
<th>100</th>
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<tbody>
<tr>
<td>pCrkl (±)</td>
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<td>eIF4E (±)</td>
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</table>

**Bead assay -- K562 cell Bcr-Abl activity**

<table>
<thead>
<tr>
<th>µM IM pretreatment</th>
<th>0</th>
<th>0</th>
<th>1</th>
<th>10</th>
<th>100</th>
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</thead>
<tbody>
<tr>
<td>100 nM IM added</td>
<td>+</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>a-p-Tyr (±)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>MC (±)</td>
<td></td>
<td></td>
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</table>

**Assay of Gleevec resistance in CML patient cells**

<table>
<thead>
<tr>
<th>µM Imatinib</th>
<th>0</th>
<th>0.1</th>
<th>1</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>50</th>
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<tbody>
<tr>
<td>K562 cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IC50 ~ 10 µM</td>
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<td></td>
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<td>IC50 &gt;&gt; 50 µM</td>
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<tr>
<td>Imatinib resistant CML patient peripheral blood ficoll-paque extracted</td>
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<tr>
<td>α-P-Tyr</td>
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</tbody>
</table>

D. Wu, D. Sher
Rapid translation to clinic…
Adapt kinase assay to Luminex technology

1) + cell extract, +ATP, +/- imatinib
2) anti-phosphotyrosine
3) anti-IgG-phycoerythrin

Well C2
Bead A mean fluorescence
Bead B mean fluorescence
Bead C mean fluorescence
Luminex bead assay for imatinib sensitivity

IC$_{50}$ ~ 20 µM

K562 extract

~10 µg lysate/well
~1 h reaction
~1 min to read

~50:1 S/N

S. Petersen
Chip based on ez-rays commercial hydrogel slide

ez-rays slides, multiwell plates (Matrix Technologies)

Activation by TCEP

Bisacrylamide

Cys-Abltide

D. Wu
High throughput Abl/Bcr-Abl activity assay

96 well ez-ray™ plates

K562 cell extract, Abtide
10 µM ATP, 1 h @ 30°C

- peptide - kinase + peptide + kinase

<table>
<thead>
<tr>
<th>µM drug</th>
<th>PD 180970</th>
<th>PD 168326</th>
<th>PD 173955</th>
<th>Imatinib</th>
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<td>10</td>
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<td>10²</td>
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<tr>
<td>10³</td>
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IC₅₀ ~ 10 µM

+ 1 µM Imatinib + 200 µM BioMol Kinase Inhibitor set

+ peptide - kinase

B6  Staurosporine  G1  Erbstatin analog  H2  Triciribine
B7  AG-494  G2  Quercetin dihydrate  H3  BML-257
C11  Piceatannol  G8  SP 600125  H4  SC-514
C12  PP1  G9  Indirubin  H5  BML-259
D9  Ro 21-8220  G10  Indirubin 3' monoxide  H6  Apigenin
F1  5-iodotubercidin  G12  Kenpaullone  H7  Erlotinib analog
F4  PP2  H1  Terreic acid  H8  Rapamycin

+ PD180970, µM 10⁻²  10⁻¹  1  10
+ Imatinib, µM 10⁻²  10⁻¹  1  10
Acrylic chemistry--Super glue for proteins

Acrylated glass

(3-acryloxypropyl)-trimethoxysilane

Acrylated protein

6-((acrylo)amino) hexanoic acid, succinimidyl ester

GFP, etc.

acrylic-labeled protein

S. Brueggemeier
Copolymerization *in situ*  →  Acrychip
Quantitative detection of Bcr-Abl inhibition by Imatinib

- ECL Average Gray Value from phosphorylated tyrosine
  - GST-Crkl (both SH3's)
  - GST-Crkl (full length)

IC$_{50}$ ~ 20 µM
Label-free detection of peptide phosphorylation

Matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry

[Diagram showing MALDI TOF process with UV laser, 25 kV, Desorption/Ionization Detector, Time-of-flight tube, and ABI 4700 machine.]

Peptide: 1357
Phosphopeptide: 1437

Intensity: 80 au
Photocleavable peptide array with MALDI read-off

1 µl spot with 10 µM Abltide, c-Abl, 1 h at 30 °C

β-NPA photolinker

Photocleavable peptide arrays

L. Parker et al.
In development: Multiplexed “lawn format” assay

Hydrogel pads in lawn or well geometry

Polyacrylamide copolymer
Abtide  EAIYAAPFAKKK-\(\beta\)NPA-Cys-acrylamide
Srtide  GEEPLYWSFPAKKK-\(\beta\)NPA-Cys-acrylamide etc..

Immunodetection

no Abtide-Cys + cAbl
100 µM Abtide-Cys + cAbl
500 µM Abtide-Cys + cAbl

~1 cm anti-pTyr "blot"

MALDI detection from copolymerized pad

20 µM Abtide-\(\beta\)NPA-Cys CHCA matrix
Linear positive mode

X. Shi
Toward an integrated assay system: Cotter lab (JHU) mini-MALDI-TOF mass spec

New assays, new geometries, new technologies

- **MethoCult methylcellulose colony forming cell assay**
  - Functional assay of growth inhibition by drugs
  - Slow, low-throughput

- **Glutathione agarose/GST fusion phosphorylation assay**
  - Simple, sensitive, robust (Stratagene SignalScout)
  - Low throughput

- **Luminex glutathione bead/GST fusion phosphorylation assay**
  - Simple, semi-quantitative, high throughput, easy multiplexing
  - Dedicated reader, low sensitivity

- **Acrylamide copolymerization GST fusion phosphorylation assay**
  - Robust, high signal to noise
  - Low sensitivity, difficult multiplexing

- **ez-rays 96 well hydrogel peptide phosphorylation assay**
  - Simple, semi-quantitative, medium throughput
  - Low sensitivity, difficult multiplexing

- **Photocleavable peptide array with MALDI read-off**
  - Robust, semi-quantitative, high throughput, easy multiplexing
  - Dedicated reader, low sensitivity
The Team & Acknowledgements

**Assays**
Steve Kron  
Ding Wu  
Xiangfu Shi  
David Rhee  
Jennifer Campbell  
Shariska Petersen

**Cells/Patients**
Wendy Stock  
Dorie Sher  
Matthew Myers

**Surface chemistry**
Sean Palecek  
Shawn Brueggemeier

**Peptides/MALDI**
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Laurie Parker  
Vivian Tien

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NSF Chicago MRSEC

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