1. Automate the determination of biochemical parameters
2. PK/PD simulations with multiple injections
EGFR inhibition by covalent drugs

Schwartz, P.; Kuzmic, P. et al. (2014)
"Covalent EGFR inhibitor analysis reveals importance of reversible interactions to potency and mechanisms of drug resistance"

PRACTICAL CHALLENGES:

**Outlier rejection**
Certain “defective” progress curves were manually excluded from analysis.

**Initial estimates**
Suitable initial estimates of rate constants were discovered by trial and error.

This “manual” method is not ideally suited for routine production environment.

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Full automation: Five passes through raw data

**Piecewise linear fit:**
Eliminate "defective" progress curves

**“Local” algebraic fit of reaction progress:**
Determine offsets and initial rates

**Algebraic fit of initial rates:**
Determine $K_i^{(app)}$ for initial non-covalent complex

**Global numerical fit of reaction progress: Pass #1**
Determine $k_{nact}$, $K_i$, and $k_{nact}/K_i$ under rapid-equilibrium approximation

**Global numerical fit of reaction progress: Pass #2**
Estimate lower limits for $k_{on}$ and $k_{off}$ under steady-state approximation
Full automation: Sharing of intermediate results

- Piecewise linear fit
- "Local" algebraic fit of reaction progress
- Algebraic fit of initial rates
- Global numerical fit: Pass #1
- Global numerical fit: Pass #2

- initial rates
- baseline offsets
- mark-up of raw data files
- lower limit estimate

Full automation: Implementation - Scripting

- Master script (Perl)
  - Perl script: QA/QC
  - Perl script: initial rates
  - Perl script: $K_i^{(app)}$
  - Perl script: $K_{\text{inact}}$ $K_i$
  - Perl script: $k_{\text{on}}$ $k_{\text{off}}$

- DynaFit
Quality control of raw data: Piecewise linear fit - Method

1. Fit progress curves to three linear segments.
2. Examine the linear slopes in each segment.
3. If the slope in either the second or the third segment is negative, reject the entire progress curve.
4. Reject also corresponding curves from remaining replicates.

Quality control of raw data: Piecewise linear fit - Results

Accept  Reject
Quality control of raw data: Piecewise linear fit - Summary

<table>
<thead>
<tr>
<th>Compound</th>
<th>I_12</th>
<th>I_11</th>
<th>I_10</th>
<th>I_9</th>
<th>I_8</th>
<th>I_7</th>
<th>I_6</th>
<th>I_5</th>
<th>I_4</th>
<th>I_3</th>
<th>I_2</th>
<th>I_1</th>
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<tbody>
<tr>
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</tbody>
</table>

Table 2.1: Acceptance results for inhibitor concentrations. “X” means that the given concentration was rejected for all three replicates. For detailed explanation see text.

**NOTE:** Each assay will require its own set of heuristic QA/QC rules!

Local algebraic fit to determine initial rates - Method

Fit fluorescence vs. time to an **exponential** equation

\[ F = F_0 + r_p [P] \]

- \( F_0 \) ... instrument baseline
- \( r_p \) ... concentration-to-signal scaling parameter
- \([P]\) ... product concentration at time \( t \)

\[ [P] = \frac{v_i}{k_{obs}} \left[ 1 - \exp\left( -k_{obs} \cdot t \right) \right] \]

- \( v_i \) ... initial reaction rate
- \( k_{obs} \) ... first-order rate constant

Reused in subsequent steps of the fully automated system
Local algebraic fit to determine initial rates - Results

<table>
<thead>
<tr>
<th>reused</th>
<th>$F_0$</th>
<th>$(-179 \pm 31)$ RFU</th>
</tr>
</thead>
<tbody>
<tr>
<td>ignored</td>
<td>$v_1$</td>
<td>$(3.23 \pm 0.18)$ RFU/sec</td>
</tr>
<tr>
<td></td>
<td>$k_{obs}$</td>
<td>$(0.0018 \pm 0.0001)$ 1/sec</td>
</tr>
</tbody>
</table>

"Morrison equation" for tight-binding enzyme inhibition:

\[
v_1 = V_0 \frac{[E]_0 - [I]_0 - K_i + \sqrt{([E]_0 - [I]_0 - K_i)^2 + 4 [E]_0 K_i}}{2 [E]_0}
\]

(4.5)

<table>
<thead>
<tr>
<th>symbol</th>
<th>unit</th>
<th>significance</th>
<th>note</th>
</tr>
</thead>
<tbody>
<tr>
<td>$v_1$</td>
<td>RFU/sec</td>
<td>observed initial rate</td>
<td>dependent variable</td>
</tr>
<tr>
<td>$V_0$</td>
<td>RFU/sec</td>
<td>initial rate at $[I] = 0$</td>
<td>adjustable parameter</td>
</tr>
<tr>
<td>$[E]_0$</td>
<td>M</td>
<td>enzyme concentration</td>
<td>adjustable parameter</td>
</tr>
<tr>
<td>$K_i$</td>
<td>M</td>
<td>apparent binding affinity</td>
<td>adjustable parameter</td>
</tr>
<tr>
<td>$[I]_0$</td>
<td>M</td>
<td>inhibitor concentration</td>
<td>independent variable</td>
</tr>
</tbody>
</table>

A little twist:

Optimize $[E]_0$ but only within a narrow range (up to $[E]_{nominal}$).
### Inductive fit of initial rates - Results

$$K_{(\text{app})} = (6.3 \pm 0.8) \text{ nM}$$

Used to make the initial estimate of $$k_{(\text{on})}$$ in global fit of progress curves

$$k_{(\text{on})} = c_{(\text{app})} \times k_{(\text{on})}$$

### Global fit of reaction progress - Method

"Generalized mechanism" (no longer simplified "Hit-and-Run" model):

- $E + T \leftrightarrow E.T : k_{aT} \leftrightarrow k_{dT}$
- $S + E.T \leftrightarrow S.E.T : k_{aS} \leftrightarrow k_{dS}$
- $S.E.T \rightarrow P + E + D : k_{\text{cat}}$
- $E + I \leftrightarrow E.I : k_{aI} \leftrightarrow k_{dI}$
- $E.I \rightarrow E-I : k_{\text{inact}}$
- $S + E.I \leftrightarrow S.E.I : k_{aS} \leftrightarrow k_{dS}$
- $S.E.I \rightarrow S.E-I : k_{\text{inact}}$
- $S.E.I \leftrightarrow S + E-I : k_{aS} \leftrightarrow k_{dS}$

DynaFit notation
Global fit of reaction progress - Results

Correlation of biochemical rate constants with cellular potency

1. Automate the determination of biochemical parameters
2. PK/PD simulations with multiple injections
Possible cellular mechanism

REALISTIC PK/PD MODEL MUST ACCOUNT FOR METABOLISM OF PROTEIN AND DRUG MOLECULES

protein re-synthesis

\[ \text{v}_{\text{synth}} \rightarrow E + I \xleftrightarrow{k_{\text{on}}} E\cdot I \rightarrow E\sim I \]

\[ k_{\text{deg}} \]

\[ k_{\text{deg}} \]

protein degradation

drug elimination

protein degradation

Possible cellular mechanism in DynaFit software

DYNAFIT USES "SYMBOLIC" REPRESENTATION OF ARBITRARY MOLECULAR MECHANISM

Example DynaFit input:

```plaintext
[task]
  task = simulate
data = progress

[mechanism]
  E + I ⇌ E.I : kon koff
  E.I → E~I : kinact
  I → X : kout
  E → X : kdeg
  E~I → X : kdeg

...```

Irreversible Inhibition Kinetics 17

Irreversible Inhibition Kinetics 18
DynaFit simulation output: Afatinib – strong inhibitor

Afatinib:
kon = 18
koff = 0.044
kinact = 0.0024

Simulate multiple injections - Method

1. Set initial concentrations of [Enzyme] and [Inhibitor]
2. Run a DynaFit simulation for one injection
3. Record concentrations at the end of the run
4. Increase [Inhibitor] concentration by next injection amount
5. Set initial concentrations to the final values (after adjusting [I])
6. Go to step #2 above
Multiple injections: Implementation - Scripting

Master script input:

\[
\begin{align*}
\text{k}_\text{on} &= 198.954 \quad ; \text{binding} \\
\text{k}_\text{off} &= 0.0472361 \quad ; \text{dissociation} \\
\text{k}_\text{inact} &= 0.0016792 \quad ; \text{covalent inactivation} \\
\text{k}_\text{elim} &= 0.0000641803 \quad ; 3 \text{ h drug half-life} \\
\text{k}_\text{psyn} &= 0.00000001605 \quad ; 0.0001 \text{ uM per 12 h * ln(2)} \\
\text{k}_\text{deg} &= 0.00001605 \quad ; 12 \text{ h protein half-life} \\
\text{E} &= 0.0001 \\
\text{EI} &= 0 \\
\text{EJ} &= 0 \\
\text{I} &= 0.01 \\
\text{ReinjectI} &= 0.01 \\
\text{Mesh} &= \text{linear from 0 to 43200 step 600} \quad ; 12 \text{ hours total} \\
\text{Injections} &= 10 \\
\end{align*}
\]

Multiple injections: Results

Simulate 10 injections @ 12 hours each:

- **Compound 2:** strong inhibitor
- **Compound 4:** weak inhibitor
Multiple injections: Results – Increase injection frequency

**Compound 5:**
intermediate inhibitor

Inject every 12 hours

Inject every 8 hours

Irreversible Inhibition Kinetics

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Multiple injections: Results – Decrease injection frequency

**Compound 5:**
intermediate inhibitor

Inject every 12 hours

Inject every 24 hours

Irreversible Inhibition Kinetics

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Simulating multiple injections: Summary and conclusions

IMPLEMENTATION:

• DynaFit does not have to be enhanced or modified to do PK/PD simulations
• PK/PD module can be implemented as a simple Perl script
• Perl scripts are simple text files: can be modified by any programmer

RESULTS (not shown):

• Association ("on") rate constants are very important for PK/PD outcome
• Dissociation ("off", "residence time") rate constants appear less important

CAVEAT: Highly reliable values for “on” / “off” rate constants are needed!