Natural Product Screening and Hit Characterization using Affinity Mass Spectrometry-Based Automated Ligand Identification System (ALIS)

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Outline

• Introduction to **Automated Ligand Identification System** (ALIS)
  – Screening tool
  – Affinity triage tool

• Application of ALIS to Natural Products (NP) Research
  – Sample suitability studies for NP extracts
  – Counter screening to isolate specific binders
  – Software tools to prevent replication
  – Triage methods to affinity rank newly discovered compounds
  – ALIS-MS/MS for structural elucidation of unknown NP hits
Automated Ligand Identification System (ALIS)

The ALIS system integrates six fundamental technologies:

- Affinity Selection
- Sample Automation
- Size Exclusion Chromatography
- Reverse Phase Chromatography
- Mass Spectrometry
- Data Analysis System
ALIS Workflow

Incubate Target protein (free in solution) with Mass-encoded libraries

Rapidly separate ligands bound to target from unbound library members by automated micro-scale SEC

Bulk Unbound Constituents to Waste

Capture complex & dissociate ligands from target; Previously Bound Ligands are analyzed by Reverse Phase LC – ESI ToF MS

ALIS software uniquely identifies ligand structure from mass information

Novobiocin
Chemical Formula: C₃₁H₃₆N₂O₁₁
Exact Mass: 612.2319

Chemical Structure:

\[
\begin{align*}
\text{H}_2\text{N} & \quad \text{O} \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{H}_2\text{N} \\
\text{HO} & \quad \text{HO}
\end{align*}
\]

22-Mar-2007
15:48:06
1. TOF MS ESI+
612.23
1.11e4

12799660011
4.36
613.15

POC: Natural Product Screening by ALIS | SPRI-Cambridge | 4
ALIS: Unique Advantages for Natural Product Screening

• High sensitivity and broad dynamic range enable drug discovery and characterization from low-level components of complex mixtures

• SEC eliminates false positives due to non-specific binding

• Accurate mass measurements yield empirical formulas for NP database searches

• MS-MS provides structural data and allows MS-triggered purification and fingerprint matching

• Affinity estimates in complex mixtures enable rapid triage
  – Insensitive to component concentration
  – Triage hits before isolation, testing, & structure determination
Proof of Concept Goal: Determine the Feasibility of Screening Natural Product Extracts with ALIS

• Well-characterized target and known inhibitor
  – Gyrase-B (a Gyrase holoenzyme subunit)
    – Well-known target for antibiotic therapy
    – Soluble protein; Well-behaved in ALIS
  – Novobiocin
    – Aminocoumarin antibiotic isolated from actinomycete in the mid-1950s
    – Binds to the Gyrase-B subunit with nanomolar affinity
    – Inhibits ATPase activity

• Experimental design
  – Linearity and limits of detection of Novobiocin
  – Sample Suitability of Natural Product extracts from multiple sources
  – Analysis of endogenous Novobiocin in active NP extracts
  – Software tools
  – Affinity rank detected components
  – Identify novel components using ALIS-MS/MS
Novobiocin Standard Quantification by LC-MS/MS

- Standard Novobiocin (0.01 – 10µM) used to generate MS-MS fingerprint & calibration curve ($R^2 = 0.99957$)

**Diagram**
- LC-MS-MS Product Ion $m/z$ 189.09
- LC-MS-MS TIC
- LC-MS Novobiocin $m/z$ 613.2
- Isobaric Noise
- 189.1032
- 189.1064

**Chemical Structure**
- NO
- O
- O
- O
- O
- O
- O
- N
- O
- O
- O
- O
- O
- O
- N
- O

**Data**
- LC-MS: NO
- NO
- O
- O
- O
- O
- O
- O
- N
- O
- O
- O
- O
- O
- O
- N
- O
- 1455.19
- TOF MS/MS ES+
- 109
- 183
- TOF MS/MS ES+
- TIC
- 400
- TOF MS/ES+
- 613.2
- 365
- 365

**Graphs**
- Annotated peaks at $m/z$ 189.09 and 613.2 with isobaric noise annotations.
Novobiocin Standard Analysis by ALIS-MS

- **K_d** determination: 0.01-125µM Novobiocin incubated with 2.0µM Gyrase-B
- ALIS binding conditions: 50 mM TRIS, pH 8.0, 40 mM KCl, 10 mM NaCl, 1 mM EDTA, 2 mM DTT, 4% glycerol.
- SEC (Agilent 1100): F = 450 µL/min, 700 mM Ammonium acetate, pH 8.0; SEC media produced in-house
- RPC (Agilent cap1100): F = 20µL/min, 0.2% Formic Acid in Water/Acetonitrile; 0.5x50mm Higgins C_{18} column
- MS: Waters LCT ESI-ToF
- ALIS limit of detection = < 0.008 µM
  - < 5 ng/mL
  - < 10 ppm of total NP extract mass
Sample Suitability of NP Extracts for ALIS: Screening Against Gyrase-B

Sample Preparation of Extracts

- 1-mg samples of NP extracts dissolved in 40 µL DMSO
  - 25 mg/mL final concentration
- ALIS sample preparation:
  - 1:20 buffer dilution, centrifugation
  - 1:1 dilution with buffer containing 5-10 µM Protein
  - Overall 1:40 dilution
- NP extract total mass = 0.625 mg/mL in 2.5% DMSO
  - 500 MW cmpd @ 100 ppm total mass = 0.125 µM (250 fmol in 2 µL)
  - ALIS limit-of-detection ≈50 fmol for most drug-like molecules

Robustness Set

- Sampling of 1500-member robustness set (no endogenous Novobiocin) was chosen at random, equally weighted to different sources
- Screened ± novobiocin at 0.5 µM
- Invertase screened side-by-side with Gyrase-B samples

SEC-UV Chromatogram

- Protein with bound ligands – transfer to RPC-MS
- Non-binding constituents transfer to waste
Sample Suitability of NP Extracts for ALIS: SEC Peak Quantification
Endogenous Novobiocin in Active NP Extracts: LC-MS/MS Quantification and ALIS-MS Screening with Gyrase-B

- Endogenous Novobiocin detected in active NP extracts at levels from 0.5 to 6 µM (0.3 to 3.6 µg/mL) after standard ALIS 1:40 dilution
- Novobiocin recovery in ALIS parallels its concentration in extracts
- Two samples purported to contain novobiocin are below LODs of both methods
- Novobiocin was not detected in active samples containing other NP
ALIS Screening of Gyrase-B against Active Extracts

<table>
<thead>
<tr>
<th>NP_extract</th>
<th>Target</th>
<th>Trunc_mass</th>
<th>measured_m/z</th>
<th>signal_strength</th>
</tr>
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<tbody>
<tr>
<td>A2_Novo</td>
<td>Invertase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gyrase B</td>
<td>599</td>
<td>599.2291</td>
<td>531.43</td>
<td></td>
</tr>
<tr>
<td></td>
<td>613</td>
<td>613.2415</td>
<td>4731.3</td>
<td></td>
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<tr>
<td></td>
<td>643</td>
<td>643.2570</td>
<td>1844.96</td>
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<tr>
<td></td>
<td></td>
<td>643.2571</td>
<td>1811.32</td>
<td></td>
</tr>
</tbody>
</table>

- Side-by-side screening of breakthrough control protein (Invertase) indicates very little SEC “breakthrough” or non-specific binding by NP extract components.
- Hits present in both samples are not of interest.
- Comparison enables identification of specific hits (e.g., novobiocin, 613) and detection of unknown entities (e.g., m/z 599 and 643).
ACE$_{50}$ Method: ALIS Competition Experiments

- ACE$_{50}$ value of the ligand is the [titrant] at which the ligand recovery is reduced by 50%.
- ACE$_{50}$ values depend on the $K_d$ of the titrant and the ligand.
- If the receptor is present in excess, the ACE$_{50}$ value is insensitive to the concentration of the unknown.
Gyrase-B ACE<sub>50</sub> Experiments: Standards and Active NP Extract

- Coumermycin is directly competitive with Novobiocin
- Two new hits detected in active extract with weaker affinity than Novobiocin
  - \( m/z \) 599.2241,
  - \( \text{Novobiocin} - \text{CH}_3 \) ?
  - \( \Delta \text{ppm} = -6.3 \pm 10.2, n = 13 \)
  - \( m/z \) 643.2503
  - \( \text{Novobiocin} + \text{CH}_3\text{O} \) ?
  - \( \Delta \text{ppm} = 0.0 \pm 11.3, n = 19 \)
ALIS-MS/MS Identification of New Hits from Active NP Extract

Novobiocin

m/z 599 A

m/z 599 B

m/z 643
ALIS-MS/MS Identification of New Hits from Active NP Extract

![Graphs showing EIC profiles and molecular structures for m/z 613, 599, and 643, along with corresponding TIC values and peak times.]

- **EIC:** m/z 613 with TIC: 2.45e3 at 4.91
- **EIC:** m/z 599 with TIC: 193 at 4.73 and 4.84
- **EIC:** m/z 643 with TIC: 619 at 4.96

Novobiocin

(A) Early peak of m/z 599

(B) Late peak of m/z 599
ALIS-based Affinity Ranking: Absolute $K_d$ Determination

- $ACE_{50}$ curves enables $K_d$ estimates
- Spike internal calibrants of known $K_d$ into mixture (e.g. NP extract)
- Compare the calibrant $ACE_{50}$ values to those of the other mixture components
- Plotting calibrant $ACE_{50}$ values versus their known $K_d$ values yields absolute $K_d$ values of unknown
Calibrants Spiked into NP Extract to Determine Absolute $K_d$ for New Hits

- By plotting known $K_d$ values vs. ACE$_{50}$ values, it is possible to calculate the $K_d$ values of the unknowns
- All 3 unknowns $K_d$ values are $\sim 0.1\mu M$
- This ALIS ACE$_{50}$ method distinguishes between two unknowns with the same molecular weight and different affinities
Conclusions

• ALIS can identify target-specific ligands from Natural Product extracts
  – Sample preparation is simple; Amenable to automation
  – Protein SEC behavior is acceptable
  – Individual component limit-of-detection is 10-100 ppm of crude extract

• Ability to select of target-specific ligands via counter-screening
  – Eliminates non-specific hits
  – Conserves resources

• Hit triage is demonstrated by ALIS ACE\textsubscript{50} experiments prior to purification and using minimal amounts of protein and crude extract
  – Determination of relative affinity
  – Accurate mass information and MS/MS characterization
  – Affinity ranking with internal calibrants allows absolute $K_d$ determination

• **Successful Proof of Concept: ALIS is a unique technology with several advantages for Natural Product drug discovery**
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