

Screening For ATP Citrate Lyase Inhibitors Using Mass Spectrometry

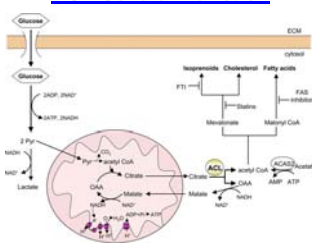
Eliza Peterson¹, Kurt Morgenstern¹, Barbara Grubinska², Can Ozbal³, William LaMarr³
¹Lead Discovery Amgen, Inc. Cambridge, MA ²Oncology Amgen, Inc. Cambridge, MA and ³Biotrove, Inc., Woburn, MA

Introduction

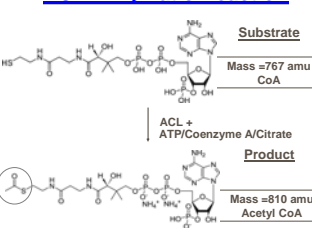
ATP-citrate lyase (ACL), a cytosolic ~400 kDa homotetrameric enzyme, catalyzes the synthesis of acetyl-CoA which is a requisite building block for *de novo* lipogenesis. Acetyl-CoA is an intermediate for malonyl-CoA and mevalonate, the committed substrates for lipogenesis. Increased levels of acetyl-CoA and the resulting *de novo* lipogenesis occurs downstream of multiple oncogenic pathways and has been shown to correlate with tumor aggressiveness [1,2]. Previous studies have demonstrated that ACL inhibition by RNAi or small molecule inhibitors dramatically reduces *in vitro* tumor cellular proliferation and tumor growth *in vivo* [3]. These results implicate ACL as a viable point of therapeutic intervention for many human cancers. In this study, we report the results of a high throughput RapidFire™ mass spectrometry assay to identify, characterize and validate ACL inhibitors. We further present our phosphopeptide mapping of protein kinase A (PKA) treated ACL using FT-Orbitrap® mass spectrometry. Moreover, the high resolution of FT-Orbitrap® was utilized for a novel proximity assay that measures the direct detection of ACL activity in cells. Using several different applications of mass spectrometry for this ACL program, we emphasize the value of mass spectrometry technology for protein and target biology programs.

[1] OskHens, et al. 1984, *Am J Physiol* [2] Savine, et al. 1967, *Cancer Res.* [3] Thompson, et al. 2005, *Cancer Cell*.

ACL Cellular Function



ACL Enzymatic Reaction



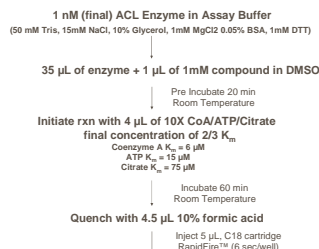
RapidFire™ Technology



RapidFire™ Highlights

- Direct detection of substrate/product
- Substrate can be lipid, peptide, metabolite, oligonucleotide, etc.
- fmol sensitivity
- 6 sec/well
- No fluorescence interference

RapidFire™ ACL Assay Summary



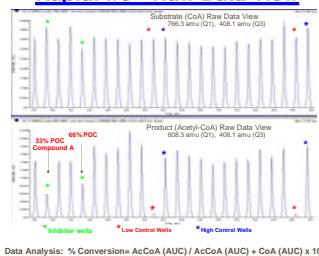
API 4000™ LC/MS/MS System
 Triple Quadrupole Mass Spectrometer
 Negative ionization and MRM Analysis

Q1: Full scan CoA - 766 amu (substrate)
 Acetyl CoA - 810 amu (product)

Q2: Fragments precursor ions

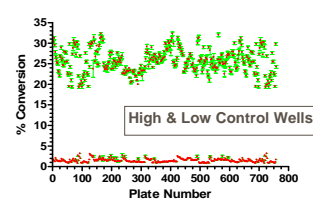
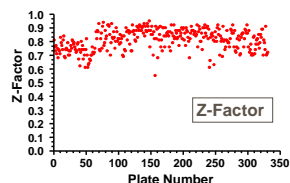
Q3: Product ions at 408 amu

RapidFire™ Raw Data View



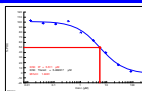
RapidFire™ Screening Results

An optimal RapidFire™ enzyme assay gave Z-factors between 0.70-0.96 and was used to screen 43,600 compounds in single point and more than 300 compounds in 10-point dose response follow-up titrations.



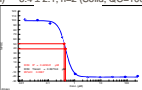
Leads from RapidFire™ Screen

Compound A



RapidFire™ HT-MS (IC50, µM) 5.9 ± 1.3, n=3 (DMSO, QC=95%)
 Transcener (IC50, µM) 6.4 ± 2.1, n=2 (Solid, QC=100%)

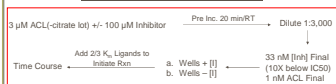
Compound B



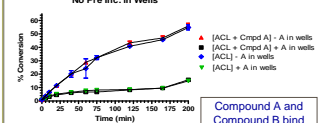
RapidFire™ HT-MS (IC50, µM) 0.43 ± 0.2, n=11 (Solid, QC=100%)
 Transcener (IC50, µM) 0.38 ± 0.04, n=2 (DMSO, QC=95%)

RapidFire™ Reversibility Studies

RapidFire™ Reversibility Study Protocol

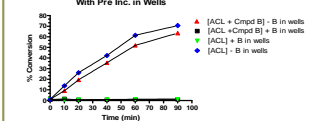


Compound A Reversibility No Pre Inc. in Wells



Compound A and Compound B bind reversibly to ACL

Compound B Reversibility With Pre Inc. in Wells

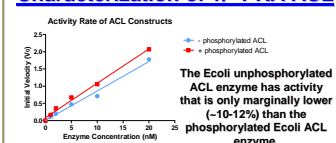


FT-Orbitrap® Technology

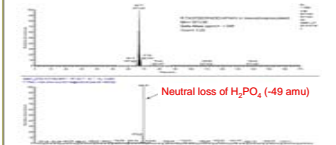


Capable of 2-5 ppm Mass Accuracy at 125,000 Resolution

Characterization of +/- PKA ACL



In contrast, Potapova, et al. reported a 6-fold increase in Ecoli expressed ACL phosphorylated by PKA (Potapova, et al. 2000, *Biochemistry*).



Phosphomapping of +/- PKA Ecoli ACL enzymes using FT-Orbitrap® MS found a tryptic peptide corresponding to residues 453-569 that is monophosphorylated (based on neutral loss of H₂PO₄)

FT-Orbitrap® Cell Assay Protocol Summary



-1X 10⁶ A549 Cells

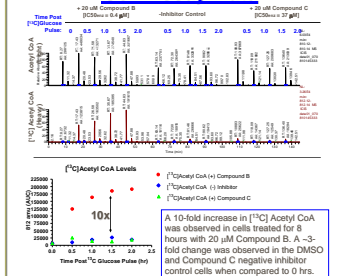
Cell Lysates

C18 Microcolumn Clean-up

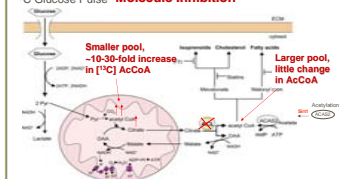
Capillary Hydrophilic C18 Column

FT-Orbitrap®

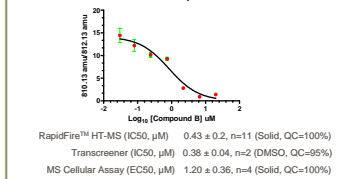
FT-Orbitrap® Raw Data View and Cell Assay Data



Hypothesis of ACL Cellular Function Upon Small 13C Glucose Pulse Molecule Inhibition



A549 Cellular Dose Response for Compound B FT-MS Cell Assay for ACL



Conclusion

Using RapidFire™ technology to identify potential ACL inhibitors, we have screened more than 43,600 compounds in single point and more than 300 IC50 follow-ups. It has been shown that the RapidFire™ platform is ideal for identifying and verifying lead compounds such as Compound A and Compound B. RapidFire™ technology was also used to look at SAR around these most potent compounds as well as investigate reversibility and probe the mechanisms of inhibition.

Using both RapidFire™ and FT-Orbitrap® mass spectrometry to characterize PKA treated ACL we phospho-residue mapped and assayed under Vmax conditions, though report only marginally lower activity with the unphosphorylated ACL enzyme as opposed to Potapova et al. who report a 6-fold increase in ACL phosphorylated by PKA.

In addition, the high resolution of FT-Orbitrap® enabled the direct analysis of ACL activity in cells and demonstrates compensatory increase in cellular AcetylCoA levels upon inhibition.

Acknowledgments

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