

- **Label-free profiling of fatty acid synthase inhibitors using a mechanistic cell-based MALDI-TOF mass spectrometry cell assay**

With the availability of Bruker's rapifleX MALDI pharma pulse for very fast matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS), MALDI-TOF MS has pushed into the field of label-free high-throughput screening in drug discovery.

Abstract

Beyond biochemical *in-vitro* assays, also phenotypic cell-based MALDI MS assays for compound profiling have been described recently. Here we

present a mechanistic cell-based MALDI-TOF MS assay for the discovery of fatty acid synthase (FASN) inhibitors. Fatty acid synthase is a central enzyme in cellular metabolism and proliferation and its dysregulation has

been linked to severe diseases. Using malonyl-CoA as a FASN specific substrate, we characterize the potency of several inhibitors described in literature.

rapifleX MALDI PharmaPulse, MALDI compound screening, cell-based assay, concentration-response, drug response marker, fatty acid synthase, mechanistic assay

Introduction

High throughput capable MALDI-TOF MS has become an emerging analytical tool in drug discovery and profiling in recent years. Mass spectrometry is a label-free technology and does, therefore, not require cost-intensive reagents such as fluorophores or antibodies and comes along without photonic artefacts like quenching, autofluorescence or spectral overlap. MALDI-TOF MS has been used as a cost effective and robust read-out for several biochemical assays in drug discovery in industrial settings [1–3]. Recently, a MALDI-TOF MS cell-based assay has been established to derive potencies of BCR-Abl inhibitors using heme B as phenotypic, metabolite marker for erythroid differentiation of K562 leukemia cells [4].

Here, we describe a mechanistic cell-based assay that monitors inhibition of the fatty acid synthase (FASN) directly in whole cells using the enzyme specific metabolite malonyl-CoA as FASN reaction read-out. The FASN is a multidomain enzyme that catalyzes the de-novo formation of the fatty acid palmitate by linking malonyl-CoA in an iterative cycle to acetyl-CoA. A variety of diseases are associated with a dysregulation of the lipid metabolism including diabetes, cardiovascular diseases and cancer [5]. Screening of published FASN inhibitors has mainly been performed using biochemical *in-vitro* assays which require large amounts of recombinantly purified FASN but cannot account for membrane permeability of the identified hits. We use a label-free MALDI-TOF MS assay with semi-automated

sample preparation, data acquisition and data analysis for the comparison of potencies of several inhibitors described in the literature.

Methods

A549 cells were cultured in RPMI-1640 medium supplemented with 10 % FCS and 2 mM L-Glutamine. For the drug response assay, cells were seeded in 96-well plates at a density of 0.1×10^6 cells per mL. After 16 hours, cells were treated with either GSK2194069, GSK837149A, Orlistat or the vehicle control DMSO for 24 hours. Cells were then harvested by centrifugation at 800 rpm, aspiration of the supernatant and snap-freezing of the 96-well plate in liquid nitrogen. Cells were resuspended at 2,500 cells/ μ L in ddH₂O supplemented with 5 μ M malonyl-¹³C₃-CoA as internal standard and two μ L from each well were transferred to a MALDI steel target in 384-format in four technical replicates. For this study, four biological replicates were assessed. Multiple assay steps have been automated including compound addition to the assay plate, sample resuspension and transfer of resuspended treated cells to the MALDI target by using a CyBio FeliX automated liquid handling robot (Analytik Jena AG, Germany) to enhance assay speed and reproducibility.

For MALDI-TOF MS, the target plate was spray-coated with DHB matrix using a TM-sprayer (HTX Technologies LLC, Chapel Hill, NC, USA). The sample plate was subsequently measured on a rapifleX MALDI-TOF mass spectrometer (Bruker Daltonics). For detailed method parameters see Table 1.

Signal validation was performed on a solariX XR 7T MALDI magnetic resonance mass spectrometer (MRMS; Bruker Daltonics).

Table 1: Sample preparation and measurement parameters

HTX TM sprayer conditions	
MALDI Matrix	20 mg/mL 2,5-Dihydroxy benzoic acid (DHB) in ACN/water (1:1 v/v) supplemented with 2.5 % TFA
Temperature	50°C
Solvent flow rate	60 μ L/min
Nozzle velocity	1000 mm/min
Line distance	2 mm
Number of passes	4
MS conditions rapifleX MALDI-TOF	
Mass range	500–1000 Da
Ion mode	Reflector positive
Laser frequency	2 kHz
Laser focus	MS thin layer
Accumulated laser shots	4000 in random walk
Sampling rate	2.5 GS/s
MS conditions solariX MRMS	
Mass range	150–5000 Da
Ion mode	Reflector positive
Laser frequency	1 kHz
Laser focus	Minimum
Accumulated laser shots	100
Scans	15
Transient length	1.47 s

After recalibration in flexAnalysis 4.0, spectra were processed and analyzed as described previously [4]. The entire MALDI-TOF workflow, including data acquisition, processing, quantitative MS feature extraction and result file generation, however, can also be controlled by Bruker's dedicated MALDI PharmaPulse software, which provides significant efficiency increases in MALDI-TOF based high-throughput screening applications. Malonyl-CoA signal intensities of technical replicates were averaged and the fold change to the vehicle control was calculated. The data was subsequently fitted

to a non-linear regression dose-response model with variable slope using GraphPad Prism 5 (GraphPad Software, San Diego, USA).

Results and Discussion

The FASN is a relevant drug target since its dysregulation is associated with several severe diseases. However, assays described in the literature rely on error prone fluorescent read-outs, require large amounts of recombinantly purified proteins and demand for extensive hit validation due to the biochemical *in-vitro* nature of the assay. Therefore, we decided

to develop a label-free cell-based assay with a high degree of assay automation and high-speed sample measurement (Figure 1). Automation steps included compound treatment of cultured cells, sample resuspension and application to 384 well MALDI target plates using a CyBio Felix automated pipetting platform in order to reduce technical variation. Resuspension of samples in a solution containing malonyl-¹³C₃-CoA as internal standard further enhanced reproducibility of the assay.

The compound GSK2194069 was described as a highly selective

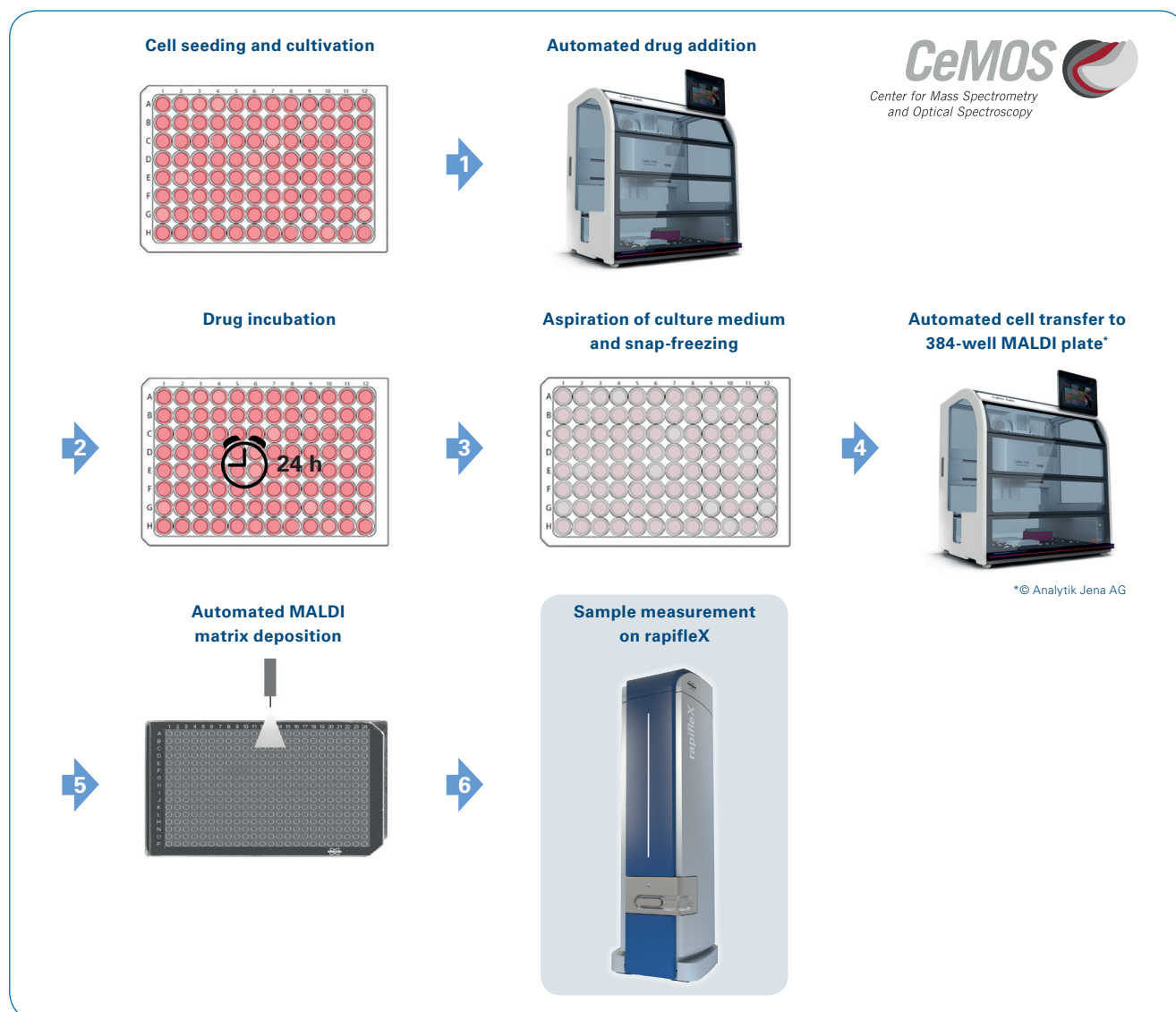


Figure 1: Sample preparation workflow for the FASN inhibitor assay

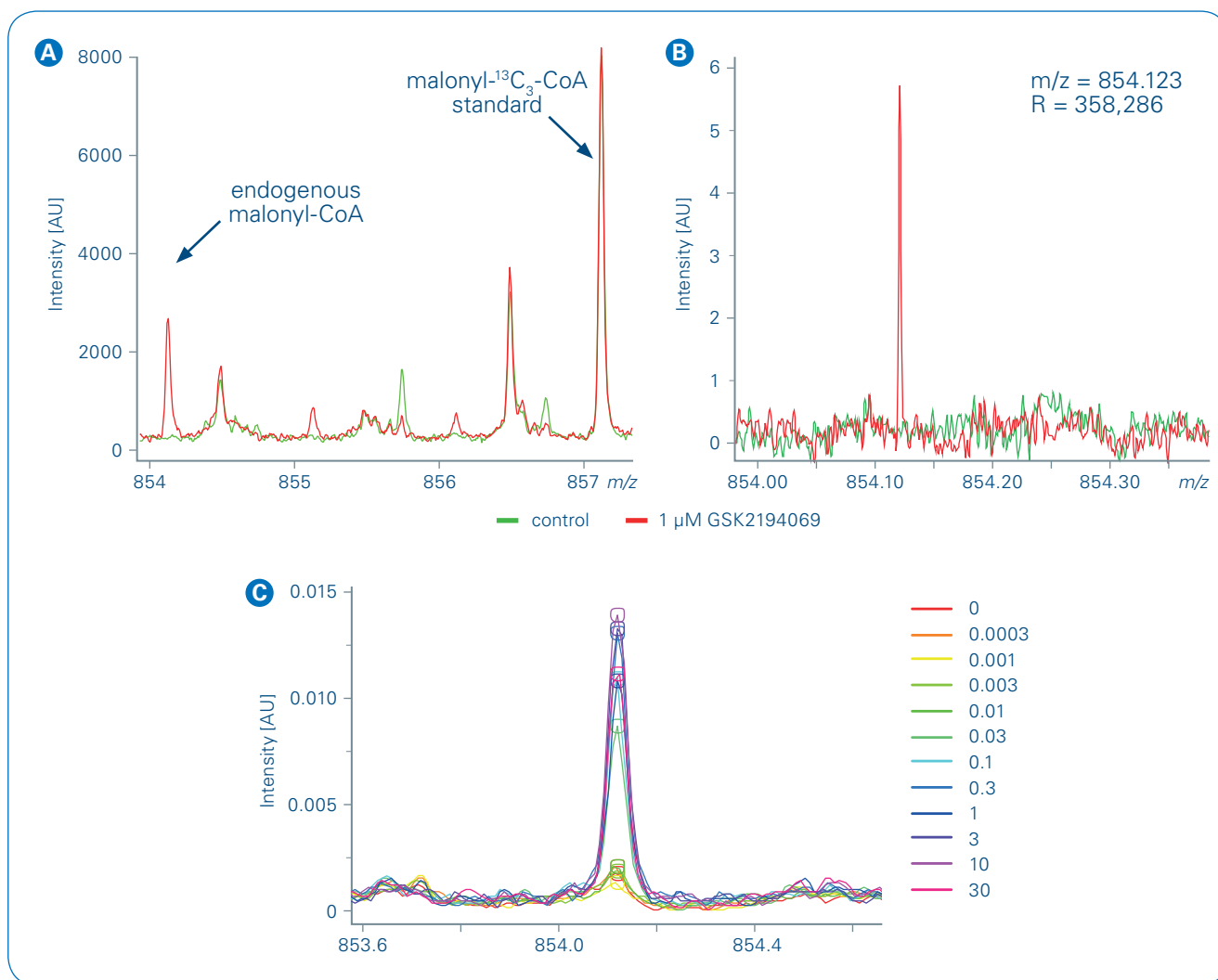


Figure 2: MALDI-TOF MS and MRMS spectra showing the specificity of the malonyl-CoA peak. (A) (B) A549 cells were treated with either GSK2194069 (red spectra) or vehicle (green spectra) and analyzed using a rapifleX MALDI-TOF MS (A) or a solariX MALDI-FT-ICR (B). (C) MALDI-TOF spectra of A549 cells treated with various concentrations of GSK2194069 and normalized to the internal standard malonyl-¹³C₃-CoA.

FASN inhibitor and was therefore used as a tool compound for assay development. Upon treatment of the A549 cells with GSK2194069, a striking increase of the endogenous malonyl-CoA signal was detected (Figure 2A). Malonyl-CoA signal validation was carried out on a solariX 7T MALDI-FT-ICR with a high mass resolution, which confirmed the sum formula of malonyl-CoA (C₂₄H₃₈N₇O₁₉P₃S) and the absence of any interfering signals (Figure 2B). Additionally, the signal intensity of the malonyl-CoA exposed a striking dependency on the administered inhibitor concentration (Figure 2C) making the FASN specific substrate

therefore a suitable pharmacodynamic response read-out for this inhibitor assay.

Next, the concentration response marker malonyl-CoA was used to compare the potency of several inhibitors described in the literature (Figure 3). Besides GSK2194069, Orlistat and GSK837149A were used for the profiling experiment. Orlistat has been described as "rather selective" inhibitor of the FASN [6]. GSK837149A was a screening hit which exposed micromolar inhibitory activity in a biochemical *in-vitro* screen against the purified, recombinant FASN [7]. However, the compound

was suspected to expose impaired membrane permeability.

Our compound profiling confirmed the strong potency of GSK2194069 with a micromolar IC₅₀ (pIC₅₀ = 7.6 ± 0.1). Also for the less selective compound Orlistat a moderate accumulation of malonyl-CoA (pIC₅₀ = 5.4 ± 0.1) was observed, confirming that Orlistat indeed inhibits the FASN. GSK837149A did not expose any inhibitory activity in our cell-based assay. This supports the hypothesis that the compound cannot pass the cellular membrane and also highlights the strength of cell-based compared to biochemical assays.

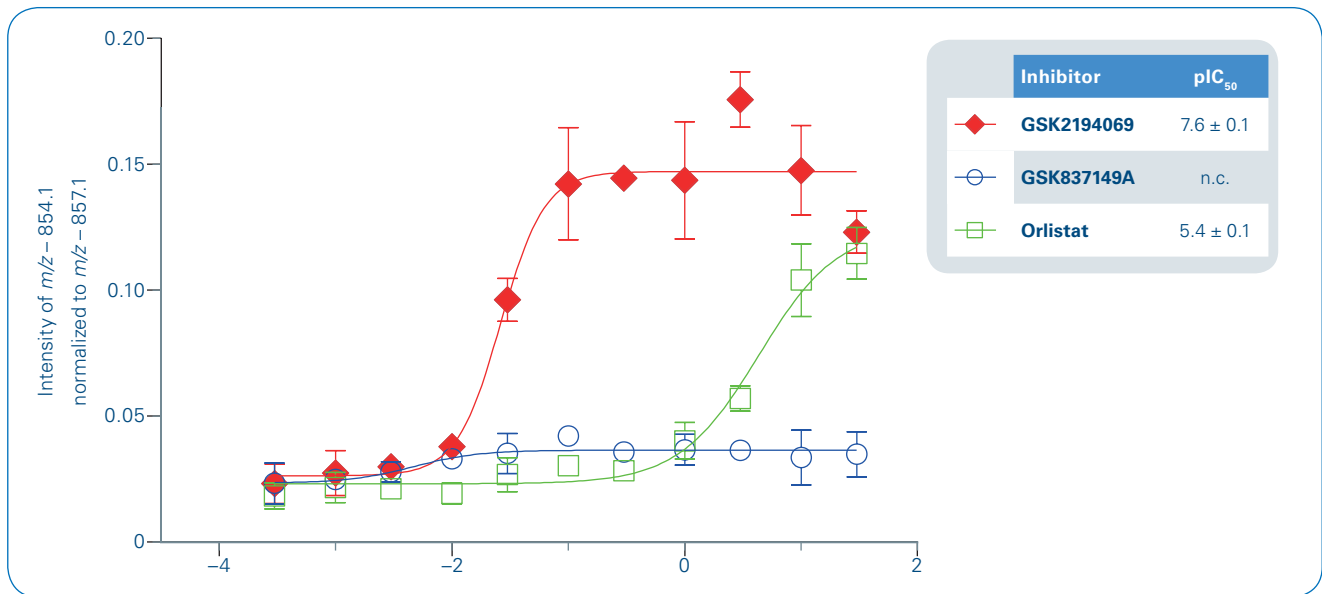


Figure 3: Quantitative MALDI MS comparison of dose responses in A549 cells.

Conclusions

- The reproducible detection of malonyl-CoA as a metabolite marker for FASN enzyme inhibition highlights the feasibility of mechanistic cell-based MALDI-TOF MS assays.
- MALDI-TOF MS assays are easily automatable using liquid handling platforms since no sample cleanup is required.
- The rapifleX MALDI-TOF MS allows for robust drug profiling and discovery at high speed.



Learn More

You are looking for further Information?
Check out the link or scan the QR code for more details.

www.bruker.com/rapiflex



References

- [1] Beeman K, Baumgärtner J, Laubenheimer M, Hergesell K, Hoffmann M, Pehl U, Fischer F, Pieck JC (2017) *Integration of an In Situ MALDI-Based High-Throughput Screening Process: A Case Study with Receptor Tyrosine Kinase c-MET*. *SLAS Discov Adv Life Sci R&D* **22**:247255521772770. doi: 10.1177/2472555217727701
- [2] Haslam C, Hellicar J, Dunn A, Fuetterer A, Hardy N, Marshall P, Paape R, Pemberton M, Resemannand A, Leveridge M (2016) *The Evolution of MALDI-TOF Mass Spectrometry toward Ultra-High-Throughput Screening: 1536-Well Format and Beyond*. *J Biomol Screen* **21**:176–186. doi: 10.1177/1087057115608605
- [3] Winter M, Bretschneider T, Kleiner C, Ries R, Hehn JP, Redemann N, Luippold AH, Bischoff D, Büttner FH (2018) *Establishing MALDI-TOF as Versatile Drug Discovery Readout to Dissect the PTP1B Enzymatic Reaction*. *SLAS Discov Adv Life Sci R&D* **23**:561–573. doi: 10.1177/2472555218759267
- [4] Weigt D, Sammour DA, Ulrich T, Munteanu B, Hopf C (2018) *Automated analysis of lipid drug-response markers by combined fast and high-resolution whole cell MALDI mass spectrometry biotyping*. *Sci Rep* **8**:11260. doi: 10.1038/s41598-018-29677-z
- [5] Menendez JA, Lupu R (2017) *Fatty acid synthase (FASN) as a therapeutic target in breast cancer*. *Expert Opin Ther Targets* **21**:1001–1016. doi: 10.1080/14728222.2017.1381087
- [6] Kridel SJ, Axelrod F, Rozenkrantz N, Smith JW (2004) *Orlistat Is a Novel Inhibitor of Fatty Acid Synthase with Antitumor Activity*. *Cancer Res* **64**:2070–2075. doi: 10.1158/0008-5472.CAN-03-3645
- [7] Vázquez MJ, Leavens W, Liu R, Rodríguez B, Read M, Richards S, Winegar D, Dominguez JM (2008) *Discovery of GSK837149A, an inhibitor of human fatty acid synthase targeting the β -ketoacyl reductase reaction*. *FEBS J* **275**:1556–1567. doi: 10.1111/j.1742-4658.2008.06314.x

For Research Use Only. Not for Use in Clinical Diagnostic Procedures.

● **Bruker Daltonics GmbH & Co. KG** **Bruker Scientific LLC**

Bremen · Germany
Phone +49 (0)421-2205-0

Billerica, MA · USA
Phone +1 (978) 663-3660

ms.sales.bdal@bruker.com – www.bruker.com