APPLICATION NOTE

#42 Utilizing Imaging Mass Spectrometry to Investigate Age-Related Renal Toxicity of RAF Inhibitor, Dabrafebnib

Application & Background

On average, only one in ten drug candidates that enters clinical phase testing is approved for human treatment.[1] While many factors contribute to the 90% failure rate of potential drugs in clinical trials, safety and efficacy are most often cited as the reasons drug candidates are abandoned. Thus, there is an obvious benefit to improved nonclinical drug analytical analyses for *in vitro* and *in vivo* studies.[1] One such methodology that is emerging in pre-clinical pharmaceutical studies is matrix-assisted laser desorption/ionization (MALDI) imaging mass spectrometry (IMS).[2] IMS allows for direct spatial correlation of histological findings and analytical molecular data. By preserving the location of analytes at a high spatial resolution, MALDI IMS can allow a better understanding of the molecular and cellular processes underlying the pathologic findings in pre-clinical drug development that could enable scientists to better anticipate and predict human risk and response to a candidate pharmaceutical.[2]

In the present study, we aimed to investigate potential age sensitivity in dabrafenib-induced renal toxicity.[2] Dabrafenib (DAB) is a competitive ATP-competitive inhibitor of RAF kinase activity that has been approved for use in adults with tumors with a *BRAF* V600E mutation.[2] However, early studies demonstrated adverse kidney effects in juvenile rats in response to DAB, including tubular deposits and dilation, cortical cysts, and tubular basophilia, although investigation into the mechanism of action of these adverse effects went beyond the scope of the original study.[3] Here, we aim to use MALDI IMS to analyze the distribution of DAB and its metabolites in the kidney tissue of juvenile rats and to determine the molecular composition of the renal deposits.[2]

Group Number	Treatment	Treatment Period	Day of Necropsy	Dose Level (mg/kg/d)
G1	Vehicle	PND 7-13	PND 14	0
G2	Vehicle	PND 14-21	PND 22	0
G3	Vehicle	PND 22-27	PND 28	0
G4	Vehicle	PND 28-35	PND 36	0
G5	Vehicle	PND 7-35	PND 36	0
G6	DAB	PND 7-13	PND 14	10
G7	DAB	PND 14-21	PND 22	10
G8	DAB	PND 22-27	PND 28	20
G9	DAB	PND 28-35	PND 36	20
G10	DAB	PND 7-35	PND 36	10/20

Table 1. Treatment conditions of 10 experimental groups.

Experimental

Study Design

All animal procedures were conducted in an American Association for the Accreditation of Laboratory Animal Care accredited facility at GlaxoSmithKine (GSK) in accordance with GSK policies on the care, welfare, and treatment of laboratory animals, and they were reviewed and approved by GSK's Institutional Animal Care and Use Committee as appropriate. Ten litters of 10 male rats were assigned to receive either the vehicle only or the vehicle + DAB.[2] Five different treatment periods and two different doses administered by oral gavage were assessed (Table 1). Groups 5 and 10 were included to match the conditions of the original juvenile toxicity study.[3] These rats were dosed at 10 mg/kg/day from post-natal day (PND) 7 to PND 21 and then increased to 20 mg/kg/day from PND 22 to PND 35. For each group, termination occurred approximately 24 hours after the last dose.[2]

Sample Preparation

At necropsy, the left kidney was bisected transversely at the hilus and kept frozen until MALDI IMS analysis.[2] Thin sections (6µm) from the midline of the kidney tissues were collected in a cryostat at -20°C and mounted onto ITO coated glass slides. Prior to matrix deposition, all tissue sections were scanned at high magnification (20x-40x) using an Aperio Scanscope CS. For a subset of animals from each group, kidney tissue homogenates were prepared from serial sections adjacent to those collected for IMS and used for LC-MS analysis.[2]

Matrix Application

DHB was applied to the slides at a concentration (C) of 50 mg/mL (in 50:50 methanol:water) using the HTX TM-Sprayer Sprayer.[2] The heated spray allowed high flow rate deposition for maximum extraction of analytes, while minimizing spatial delocalization. The total sample preparation spraying took 12 minutes per ITO slide (25x75mm).[2] The slides were coated using the following parameters:

Flow Rate (FR)	0.100 mL/min
Spray Nozzle Velocity (V)	1.333 mm/min
Spray Nozzle Temperature	70°C
Track Spacing (TS)	3 mm
Number of Passes (NP)	8

Matrix density (W) $W = \frac{NP \times C \times FR}{V \times TS} = 0.010\ mg/mm^2$

Linear Flow Rate (LFR) $LFR = \frac{FR}{V} = 7.5e\text{-}5\ mL/min^*$

* Corresponds to dry spray, defined by LFR between 8.3e-5 and 8.3e-6

MS Analysis

LC-MS quantification was performed using a Thermo Orbitrap XL.[2] MS imaging experiments were performed using a Bruker Solarix 7T Fourier transform-ion cyclotron resonance mass spectrometer (FT-ICR MS). Images were acquired at spatial resolutions ranging from 10 to 100 µm. Mass spectra were acquired both in full scan mode (m/z 80-1000) and continuous accumulation of selected ions (CASI) mode for the mass range of m/z 460-620 in order to enhance sensitivity for DAB-related material. In order to estimate tissue concentration of DAB-related material by MALDI IMS, a tissue mimetic model was used.[2] A tissue mimetic model consists of homogenate tissue cores spiked with known concentrations of the analyte of interest.[4] These cores are then analyzed under the same conditions as the tissue of interest so the analyte quantity in the tissue can be estimated from comparing the ion intensity between the calibration curve of the mimetic tissue model and the MALDI images of the samples.[4] All ion images were generated using FlexImaging v4.0 software from the raw data.[2]

Results

Histological Findings

Rats exposed to DAB only pre-weaning (Groups 6, 7 and 10) were found to have a high incidence of tubular deposits, while groups dosed only post-weaning (Groups 8 and 9) had no incidence of tubular deposits. Although dosed for a longer period of time, Group 10 had a similar density of tubular deposits to Groups 6 and 7, suggesting the cessation of tubular deposit formation around PND 22, despite continued DAB treatment. No tubular deposits were noted in the control animals (**Figure 1**).

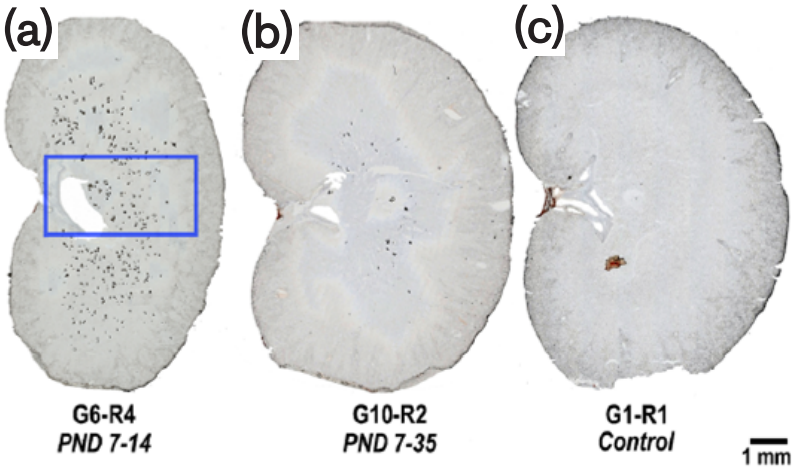


Figure 1. Optical scan of kidney tissue sections from juvenile rats: (a) Group 6 Rat 4, (b) Group 10 Rat 2, (c) Group 1 Rat 1.

Quantification of DAB Metabolites by LC-MS and Mimetic Tissue Model

The youngest rats of Group 6 were found to have the highest concentration of DAB metabolites present in the kidney by LC-MS analysis. Of the three metabolites of DAB, carboxy-Dabrafenib (CDAB) was found to be the predominant species in the kidney of the Group 6 (PND 7-14) rats. By comparing the tissue mimetic model to the average ion signal detected by MALDI IMS for an analyte in a tissue section, the estimated the concentration of CDAB in for two rats in Group 6 were 8,000 ng/g and 6,400 ng/g, respectively (**Figure 2**). LC-MS of tissue homogenates found the CDAB concentration in the kidneys of these two rats to be 11,600 ng/g and 6,100 ng/g, respectively. These data support the tissue mimetic model as a reliable method to quantify analytes in MALDI imaging, especially when considering that MALDI IMS results are generated from a single tissue section (<1 mg) while LC-MS results are generated on half-kidney extracts (>200 mg). In addition, the differences could be partially attributed to regional concentration differences of analytes in the kidney.

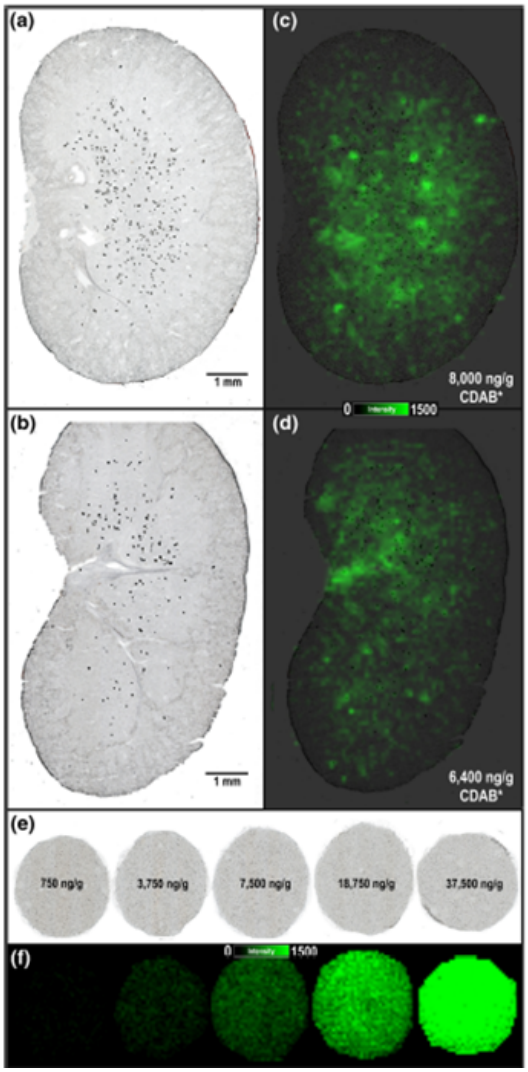


Figure 2. Optical scans of kidney tissue sections from PND 7-13 juvenile rats (a) Group 6 Rat 2 and (b) Group 6 Rat 3 analyzed by MALDI IMS in CASI mode (*m/z* 460-620) at 100 µm spatial resolution. (c) and (d). Respective ion images for CDAB (*m/z* 508.1083) and quantity (ng/g of tissue) predicted using tissue mimetic model. (e) Pre-analysis optical scan of tissue mimetic model cores with spiked concentration of CDAB labeled for each (f) ion image for CDAB (*m/z* 508.1083) from tissue model analyzed under the same conditions as the kidney tissue sections.

References

[1]*Van Norman, G. A. (2016). Drugs, Devices, and the FDA: Part 1: An Overview of Approval Processes for Drugs. JACC: Basic to Translational Science, 1(3), 170–179. https://doi.org/10.1016/j.jacbts.2016.03.002*

[2]*Groseclose, M. R., Laffan, S. B., Frazier, K. S., Hughes-Earle, A., & Castellino, S. (2015). Imaging MS in toxicology: An investigation of juvenile rat nephrotoxicity associated with dabrafenib administration. Journal of the American Society for Mass Spectrometry, 26(6), 887–898. https://doi.org/10.1007/s13361-015-1103-4*

[3]*Laffan, et al. Manuscript in preparation.*

[4]*Groseclose, M. R., & Castellino, S. (2013). A mimetic tissue model for the quantification of drug distributions by MALDI imaging mass spectrometry. Analytical Chemistry, 85(21), 10099–10106. https://doi.org/10.1021/ac400892z*

Analysis of Renal Distribution of DAB Metabolites by MALDI IMS

Low-resolution MALDI IMS (100 µm) of kidney tissues demonstrated that CDAB was most concentrated near the tubular deposits found in the inner medulla and pelvic regions of the kidney of the youngest rats (**Figure 3**). Higher resolution MALDI IMS of the medulla of a Group 6 rat shows that CDAB is specifically localized to the lumen of the damaged collecting ducts, but no DAB metabolites were found directly from the tubular deposits themselves (**Figure 3**).

Analysis of Molecular Composition of Tubular Deposits by MALDI IMS

In order to elucidate the mechanism by which pre-weaning exposure to DAB contributes to the formation of tubular deposits, it was necessary to analyze the composition of these deposits. Specifically, since no DAB metabolites were found directly to comprise the tubular deposits that formed after pre-weaning exposure to DAB, the molecular process by which these tubular deposits are formed could provide critical insight into potentially age-sensitive drug toxicities. High resolution MALDI IMS revealed that the tubular deposits were clusters of several DHB-calcium compounds (**Figure 4**).

Conclusions

MALDI IMS is an emerging analytical technique that can help toxicologists associate pathological findings with molecular biology.[2] By understanding the mechanism of action of a drug's toxicity, safety risks to humans can be better assessed before the clinical phase of drug development. In the present study, age sensitive nephrotoxicity to BRAF inhibitor DAB was demonstrated in pre-weaned juvenile rats. While it was known that younger rats have less efficient biliary clearance than older rats, these MALDI IMS data provided several insights into the potential mechanism of age-related renal toxicity of DAB. First, it was confirmed that the histologic nephrotoxicity was spatially associated with higher concentrations of DAB metabolites. Second, it demonstrated that the tubular deposits are not themselves comprised of DAB or its metabolites, but calcium salts. Third, it was found that CDAB primarily localized to the lumen of damaged collecting ducts, which are the primary site of calcium regulation in the kidney, suggesting a potential causal link between the pathologic observation of tubular deposits and DAB pre-weaning exposure.[2]

Figure 4. (a) Optical scan of kidney tissue sections from Group 6 Rat 4 analyzed by MALDI IMS in full scan mode (*m/z* 100–1000) at 10 µm spatial resolution. (b) Ion image for [3DHB – H + Ca]+ (*m/z* 501.0339) in green, and glycerophosphocholine [M + K]+ (*m/z* 296.0659) in red.

The tissue images and MS data presented in this note were provided by Dr. Reid Groseclose and Dr. Steve Castellino from the Department of Bio-Imaging of GSK, 709 Swedeland Road, King of Prussia, PA 19406, USA.

Experimental Summary

Tissue Type	Rat kidney
Preservation	Fresh frozen
Tissue Cut	6 µm

Instrumentation and Supplies

Microtome	Leica CM3050s
LC-MS	Thermo Orbitrap XL
MALDI Plate	ITO coated glass slides
Matrix	DHB (Sigma Aldrich, 50 mg/mL in 50:50 Me/H_2O)
Matrix Sprayer	HTX TM-Sprayer™
MALDI MS	Bruker Solarix 7T FT-ICR
MALDI Laser	
Acquistion Mode	Fullscan (*m/z* 800-1000) and CASI (*m/z* 460-620)
Imaging Software	FlexImaging v4.0

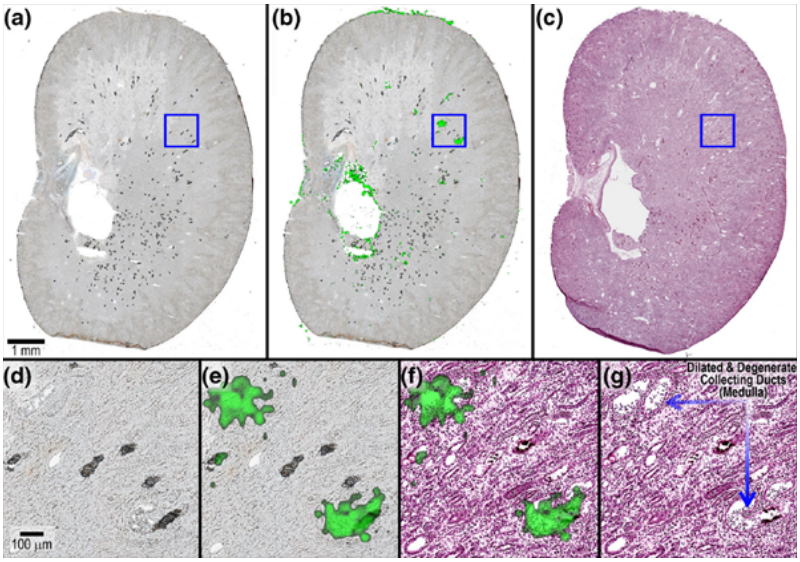
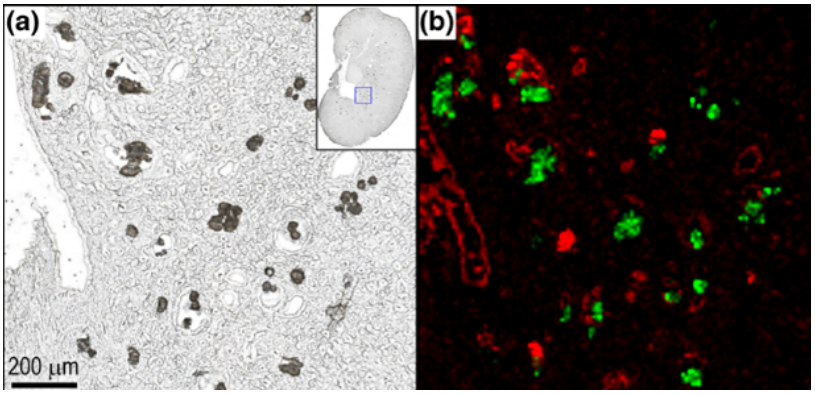
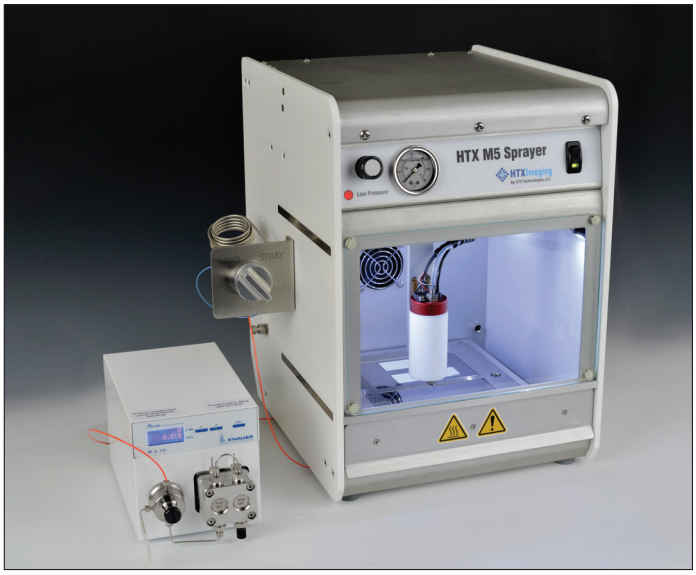


Figure 3. (a) Optical scan of kidney tissue sections from Group 6 Rat 2 analyzed by MALDI IMS in CASI mode (*m/z* 460–620) at 25 µm spatial resolution. (b) Ion image for CDAB (m/z 508.1083). (c) Serial H&E. (d) 10× magnification of outlined region in optical scan. (e) Magnified view of CDAB ion image co-registered with optical scan. (f) Magnified view of CDAB ion image co-registered with H&E. (g) Histopathology annotated H&E



HTX M5 Sprayer™ Tissue MALDI Sample Preparation System



HTX M5 Sprayer™ System is an Automated MALDI Matrix Deposition System Offering **High Reproducibility** and **Superior Data Quality** for Imaging Mass Spectrometry

The HTX M5 Sprayer™ is an easy-to-use, versatile spraying system that provides automated processes for sample preparation in imaging mass spectrometry.

The proprietary spray technology of the HTX M5 Sprayer™ guarantees a very fine, uniform and consistent matrix coating crucial for high-resolution imaging and relative quantification of analytes.

The unique ability to control liquid and propulsion gas temperature creates a fine solution mist that can be deposited in a precise and adjustable pattern over all or part of any MALDI plate.

Spray characteristics (wet or dry) are easily adjustable via the intuitive operator interface. Users can create and save methods for reproducible operation.

Key Characteristics

- Proprietary technology providing very small matrix droplets (<5 microns)
- High flow rate and fast sample prep (2 to 18 minutes per slide)
- Highly consistent matrix deposition across entire sample area (+/- 3% by weight)
- Unique use of temperature and nitrogen flow to control evaporation rate and matrix crystal formation
- More than 30 validated protocols covering trypsin and most matrices (e.g.: SA, CHCA, DHB, DAN, 9-AA, DHA, CMBT, THAP)
- Validated protocols for Trypsin digestion of FFPE
- Continuous matrix coverage as needed for high-resolution imaging
- Rugged operation and easy clean-up

Addressing the Matrix Deposition Challenge

The main challenge when preparing samples for MALDI Mass Spectrometry Imaging is to balance the positive effects of the matrix solution penetrating the tissue and co-crystallizing with the analyte, and the negative effects of analytes delocalization.

The all-new M5 chassis, high velocity stage and heated sample holder drawer contribute to a greater user experience and expanded process capabilities including:

- Faster and drier deposition capability
- On-tray trypsin digestion capability
- On-tray sample re-hydration

HTX M5 Sprayer™ is available worldwide exclusively from HTX Technologies, LLC.

To request further information contact:

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HTX Technologies offers innovative sample preparation systems for advanced analytical platforms. Our integrated workflow solutions include user training, instruments, software, consumables and method development services.

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