### BREATH BIOPSY

# High sensitivity detection of deuterium-labelled volatile compound probes with Orbitrap-MS: from in vitro headspace to Breath Biopsy<sup>®</sup> for lung cancer

#### At a Glance:

- Exogenous volatile organic compound (EVOC<sup>®</sup>) Probes were designed to target the metabolic pathways altered in lung cancer and release unique volatile reporter compounds which can be related to disease presence.
- From proof-of-concept in vitro and in vivo studies through to human clinical trials, this TD-GC-Orbitrap-MS workflow can be used to measure deuterated VOCs at low concentration in a variety of biological matrices, including breath.
- Through administration of EVOC Probes, metabolic pathways could be utilised to generate sensitive and specific on-breath signals for lung cancer.

#### **1. Background and Objectives**

Owlstone Medical is exploring the possibility to test for lung cancer and other diseases on breath using an EVOC strategy. This comprises the delivery of a probe synthesized with a cleavable EVOC reporter. Upon reaction with a pathologically relevant enzyme the reporter is released and detectable on breath.

Many enzymatic pathways are altered by lung cancer. β-glucuronidase can cleave EVOC Probe 1, which links a sugar molecule to deuterated ethanol (D5-ethanol). The released D5-ethanol can then be detected on breath samples with low background signal. We hypothesize that more probe cleavage will take place in lung cancer cases where  $\beta$ -glucuronidase is expressed in the extracellular space, as EVOC Probe 1 cannot readily penetrate healthy cells.

In addition, aldoketoreductase enzymes (AKRs) are upregulated in lung cancer in a characteristic response to lipid peroxidation. These enzymes convert aldehydes and ketones to alcohols which are more easily cleared from the body. By administering a known amount of aldehyde and monitoring for aldehyde and the alcohol bioproduct on breath, we aim to distinguish different levels of AKR activity.

These EVOC strategies have been used in pre-clinical *in vitro* and *in vivo* studies and in human trials, with the use of isotopically labelled VOCs to maximize signal-to-noise. Here we discuss optimization of the thermal desorption – gas chromatography – mass spectrometry (TD-GC-MS) method and most recent results.

Figure 2: The EVOC Probe approach. An administered EVOC Probe is introduced and monitored non-invasively on breath along with its metabolic bioproducts. Aldo-keto reductases (AKRs) are a potential EVOC Probe target that are upregulated in some cancers as an adaptation to oxidative stress and lipid peroxidation.





Figure 3: A549 and H460 lung cancer cell lines treated with chemical AKR inhibitors Tolrestat and JF0064 for 24 hours. Aldehyde substrates added to wells and supernatant collected after 0.5, 1 and 3 hours for headspace analysis. 50 µL supernatant transferred to 10mL headspace vial and sampled with High Capacity Sorptive (HiSorb) probes at 37 °C for 30 min.









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Figure 4: Large volume breath sampler used in clinical trials to collect breath samples onto sorbent tubes for

Figure 5: Mouse breath sampling device. Controls: Internal standard without and with target VOC spiked into box to demonstrate recovery through device. Un-treated healthy mice with target VOC spiked into box to demonstrate impact of animal presence on recovery. Positive control: healthy mice treated with target VOC. Tests: healthy mice and mice with cancer treated with EVOC Probe 1. All samples collected onto sorbent tubes under flow for 30 min.

# **3. Analytical Workflow**



## **4. Method Development**





**Figure 7:** Example mass spectra for naturally occurring and isotopically labelled nonanal

D5-ethanol resolution optimisation – GC1 ramp — GC2 ramp – GC3 ramp D5-ethaho D5-ethanol MTPx10<sup>-</sup> Retention time (min)

Figure 10: (Left) Pilot analysis of mouse breath sample showed high background (black trace). Split ratio applied during focussing trap desorption optimised to balance background (blue trace) and sensitivity shown in calibration curves (right).





HiSorb probes analyzed with automated analyzed with TD100xr autosampler to therm desorb VOCs. Samples pre-concentrated on cold tenax-carbograph focussing trap to allow purging of water solvent. Focussing trap desorbed at high temperature onto a Stabilwax GC column for separation. Column held at 40 °C, heated to 250 °C and held at 250 °C. Detection with Q-Exactive Orbitrap mass spectrometer in Electron Ionisation mode at 70 eV, mass range 30 - 200 m/z, single ion monitoring (SIM) scan for D5-ethanol (49), D2-trans-2-hexenol (69), D2-trans-2-hexenal (85), D18-nonanal (90) and D18-nonanol (62).









Figure 9: Optimizing resolution between target D5-ethanol 6000 and closely eluting interferent contaminants methylthiopropane (MTP) and dichloromethane (DCM) using



#### 5. Results



Figure 11: Effect of AKR inhibitors in lung cancer cells detected using aldehydes as EVOC Probes. No significant difference is observed in the analysis of substrate abundance; however, a dose-dependent response was observed between Tolrestsat and the inhibition of hexanol and trans-2-hexenol production in A549 and H460 cells treated with 10  $\mu$ M hexanal or trans-2-hexenal, respectively, after 30min. JF0064 also inhibited the AKR activity, with different levels of inhibition observed depending on aldehyde substrate and cell line. Data normalized by vehicle treatment.



increased as target VOC dosage to positive control mice increased.

### 6. Conclusions

The authors thank all study subjects and all research staff of the participating hospitals. The authors declare no competing financial interest

received a dose of EVOC Probe 1 intravenously. Breath samples were collected at 9 timepoints, including before dosage. LOD for D5-ethanol on breath: 12ppq.

• In-vitro studies show enzymatic activity relevant to lung cancer can be distinguished with VOC detection using headspace TD-GC-MS.

• In-vivo studies show reporter VOCs can be collected and detected in the same analytical workflow.

• Clinical samples analysed using the optimised TD-GC-MS method which had on-breath D5-ethanol LOD of 12ppg showed D5-ethanol reporter VOC was detectable on breath after intravenous administration of EVOC Probe 1.