BREATH® BIOPSY

Targeting tumour-associated aldo-keto reductase activity with exogenous volatile organic compound (EVOC) Probes to detect lung cancer.

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Aims

Investigate aldo-keto reductase (AKR) activity in lung cancer cells as an initial step towards developing an EVOC® Probe-based breath test for cancer screening and early detection by:

- Detecting AKR-associated alcohols and aldehydes from *in vitro* headspace using gas chromatography-mass spectrometry (GC-MS) workflow as used from clinical breath samples.
- Investigate the time-dependent impact of AKR inhibition on VOC levels in headspace samples.

1. Background and Objectives

Cancer metabolism represents a promising and largely untapped focus for diagnostic testing. What's more, while the genomics of cancer can vary extensively, these changes converge onto key metabolic pathways bringing about survival benefits that enable cancer cells to survive in the harsh tumour microenvironment.

The use of agents such as UDP-glucose in PET scans serves to illustrate how we can target metabolic pathways with exogenous compounds to provide high sensitivity cancer detection. Our hypothesis is that a similar approach can be used in combination with non-invasive breath sampling to provide reliable early detection of cancer in a form that is well suited to the type of screening programme that could dramatically improve cancer survival.

Lung cancer is an area where there is significant unmet need to improve early detection through screening of at risk populations. Incidence and mortality in these cancers is high and the majority of cases are diagnosed in the later stages. In addition, the location of lung cancers makes it particularly well suited for detection via breath sampling. As such, we have chosen to make this the initial focus of our work.

Rapid growth, poor blood flow and persistent genetic errors in cancer cells contribute to a high level of oxidative stress characterized by an increase in reactive oxygen species (ROS). In turn, ROS promote destructive processes such as lipid peroxidation which produce aldehydes. Human lung cancers increase expression of aldo-ketoreductase (AKRs) enzymes to help process these excess aldehydes and reduce them to alcohols.

We are seeking to investigate whether AKRs can be targeted using an exogenous volatile organic compound probe (EVOC® Probe), the metabolism of which can be monitored on breath using Breath Biopsy.

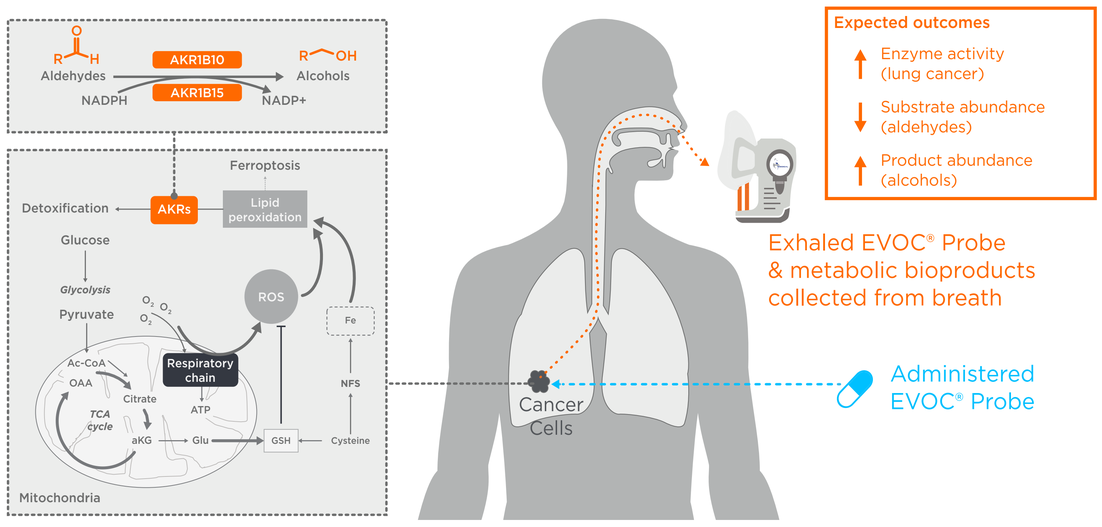

Figure 1. The EVOC Probe approach. *An administered EVOC Probe is introduced and monitored non-invasively on breath along with its metabolic bioproducts. Aldo-keto reductases (AKRs) are a potential EVOC Probe target that are upregulated in some cancers as an adaptation to oxidative stress and lipid peroxidation.*

2. Method

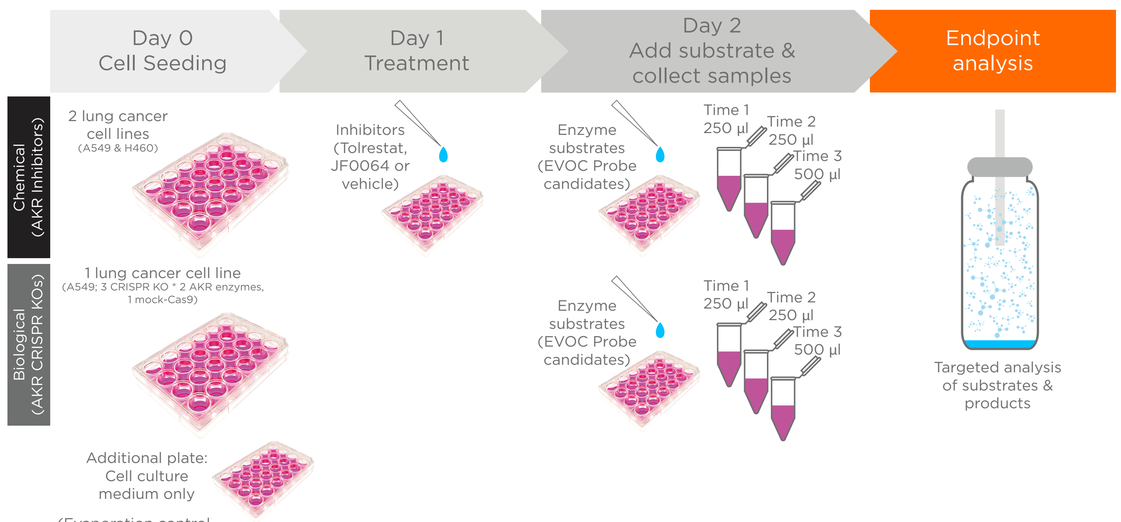

Figure 2: Method overview of biological and chemical* in vitro *methods used to investigate the effect of AKR activity modulation on released headspace VOCs. *For inhibition of the catalytic activity of AKR1B10/B15 two lung cancer cell lines were treated for 24h with two AKR inhibitors (Tolrestat: IC50 and/or IC75 based on AKR activity assay; JF0064: 10µM). For permanently abrogated AKR1B10 or AKR1B15 expression, three clones of AKR1B10 knockout and three clones of AKR1B15 knockout in the background of A549 cells were developed using CRISPR-Cas9 system. A Mock-Cas9 was also used as control (wild-type cells). Cells were treated with 10µM of aldehydes (30 µM for Alkanal 2), and aliquots of cell culture media were collected at 3 timepoints for headspace analysis. Samples were stored at -80°C before analysis. An additional plate was run in parallel in each experiment for the control of evaporation and/or cross-contamination. Aldehydes were added to this plate following the plate layout of each experiment set.*

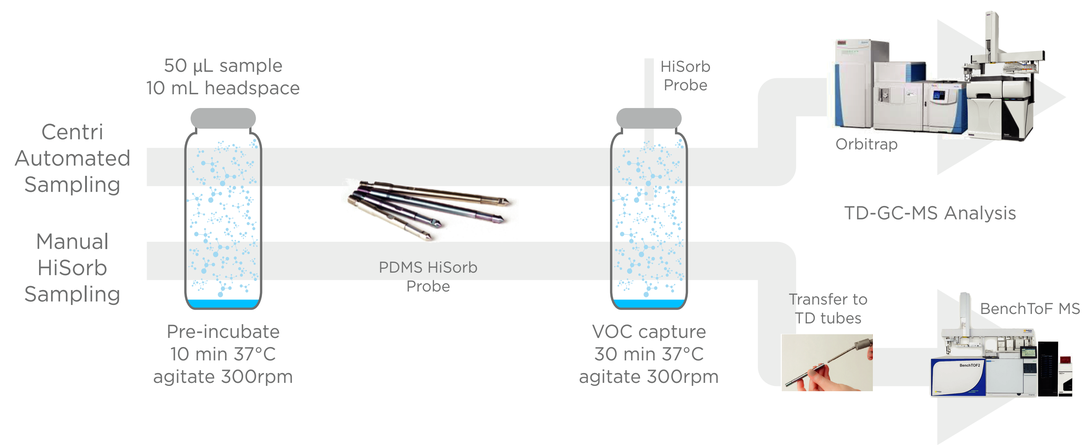

Figure 3: Automated and manual headspace VOC sampling methods coupled with a mass spectrometry system. *50 µL of aqueous samples containing the volatiles are used for headspace analysis. Quality control mix (Deuterated standards and a panel of aldehydes and alcohols) are injected onto sorbent tubes and run throughout the sequence. The mass spectrometry method is selected based on the higher sensitivity and specificity for each pair of aldehydes and alcohol products.TD-GC-MS: Thermal desorption-gas chromatography-mass spectrometry. TOF-MS: Time-of-flight mass spectrometry.*

3. Results

Overall, headspace from A549 and H460 samples showed lower levels of aldehydes and more alcohol products than those observed in evaporation controls confirming that AKRs are active in the studied lung cancer cells (Figure 6-7).

Although no significant difference is observed in the analysis of substrate abundance, our study shows that AKR1B10 and AKR1B15 are responsible for the reduction of multiple aldehydes in alcohol products in lung cancer cells (see representative images Figure 6-7).

Table 1: Linearity, LOD and LOQ of aldehydes and alcohol bioproducts in PBS using PDMS HiSorb probes. *High sensitivity and broad linearity range using both Centri automated sampling coupled with Orbitrap and manual HiSorb sampling followed by BenchTOF-MS analysis. Results were reproducible using different cell culture media. LOD: limit of detection; LOQ: Limit of quantification.*

	Manual HiSorb / benchTOF			Centri / Orbitrap		
Compound	Linearity (R^2)	LOD (µM)	LOQ (µM)	Linearity (R^2)	LOD (µM)	LOQ (µM)
Alkenal 1	0.9916	0.03	0.08	-	-	-
Alkenol 1	0.9543	0.15	0.45	-	-	-
Alkanal 1	0.8260	0.13	0.39	0.9849	0.14	0.43
Alkanol 1	0.9917	0.03	0.08	0.9884	0.04	0.13
Aromatic Aldehyde 1	0.9186	0.08	0.25	0.9835	0.07	0.22
Aromatic Alcohol 1	0.9165	0.47	1.42	0.9574	0.16	0.48
Alkenal 2	0.9291	0.96	2.90	0.9916	0.05	0.14
Alkenol 2	0.9190	0.29	0.89	0.9695	0.15	0.46
Alkanal 2	-	-	-	0.9711	0.840	2.546
Alkanol 2	-	-	-	0.9788	0.307	0.931

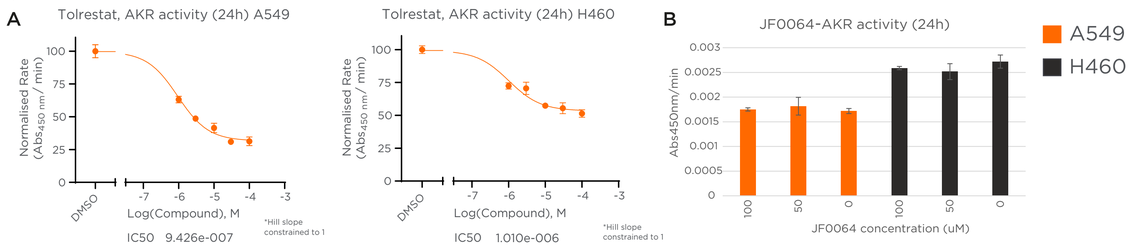

Figure 4: AKR inhibitor effects in lung cancer cells. *A commercial colorimetric assay validated to AKR1B10, AKR1C1 and AKR1C3 activity analysis was used. A) Dose-dependent effect of Tolrestat in AKR activity in A549 and H460 after 24h of treatment. B) JF0064 does not affect overall AKR activity after 24h of treatment in A549 and H460 cell lines.*

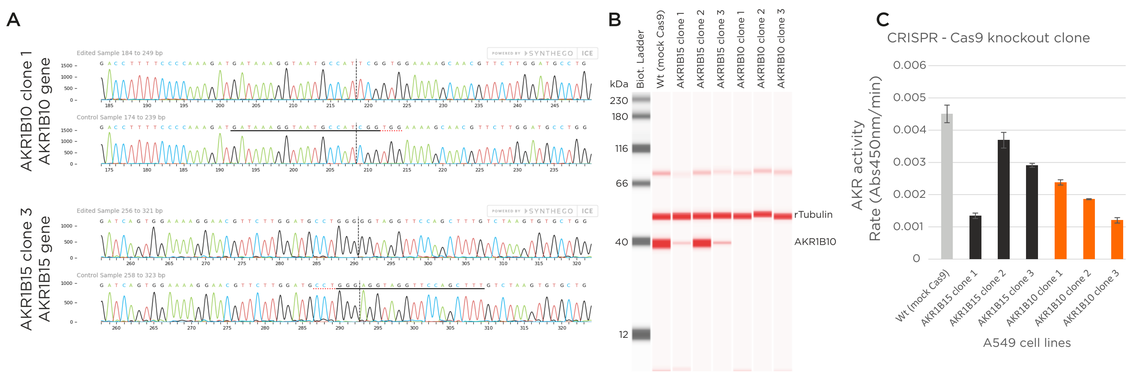

Figure 5: A549 AKR1B10 and AKR1B15 knockout cells. *A) Representative images of Sanger sequencing used to confirm the presence of indels in the AKR1B10/5 knockout; B) Western blot analysis showing that two AKR1B15 knockouts (without AKR1B10 gene edition) show downregulation of AKR1B10 protein expression. C) Using a commercial colorimetric AKR activity assay (validated with AKR1B10, AKR1C1 and AKR1C3), a reduction in AKR activity of over 50% was detected in AKR1B10 knockouts and variable levels were observed in AKR1B15 knockout. The clone without reduced expression of AKR1B10 showing AKR activity more similar to wild-type cells.*

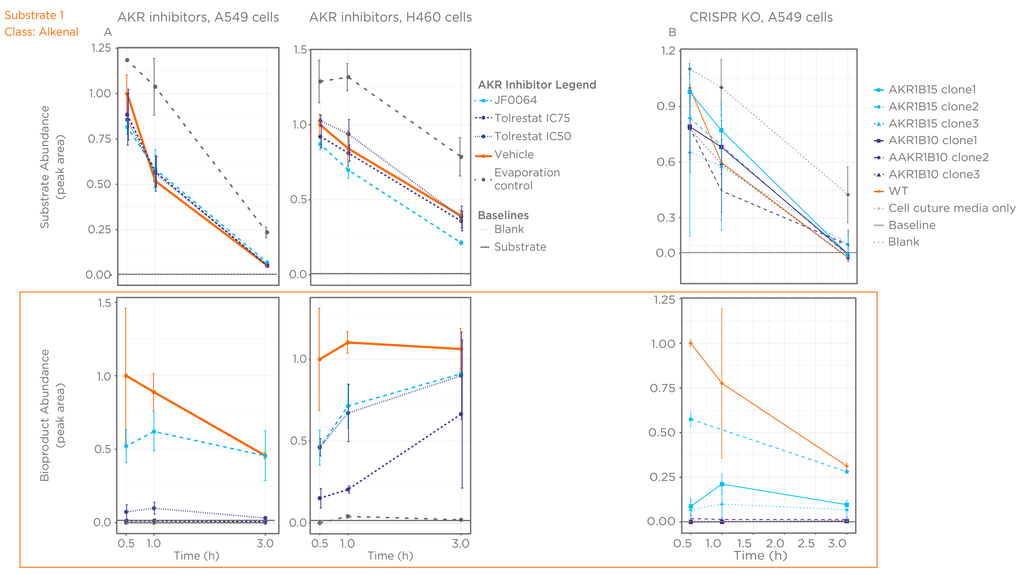

Figure 6: AKR1B10 and AKR1B15 are responsible for the reduction of an alkenal in its alkenol bioproducts in lung cancer cells. *A) The AKR inhibitor JF0064 reduced about 50% the production of alkenol and a dose-dependence response was observed between Tolrestat and the inhibition of alkenol production in A549 and H460 treated with alkenal 10 µM. B) Knockout of AKR1B10 abrogated the production of alkenol under alkenal administration (10 µM). AKR1B15 silencing also reduced the production of alkenol compared to wild-type cells. Blank: cell culture media only (no aldehydes added). Baseline: mean of analysis using cell culture media (no aldehydes added) from cell lines with/without inhibitor (A) or different clones (B). Data normalized by vehicle/WT group (first timepoint).*

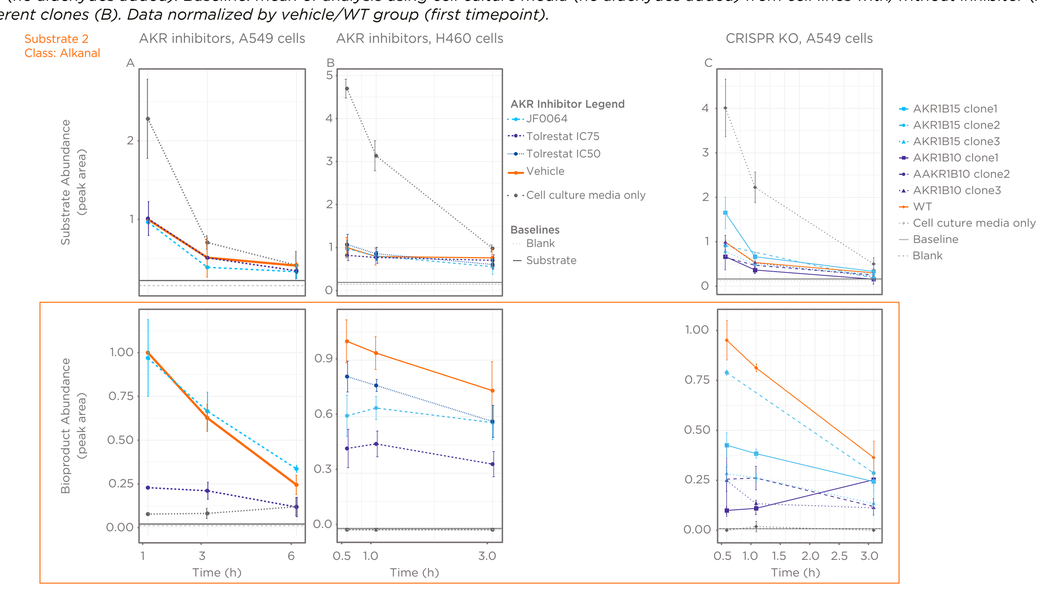

Figure 7: AKR1B10 and AKR1B15 are responsible for the reduction of an alkanal in its alkanol bioproducts in lung cancer cells. *A) Tolrestat inhibited the alkanol production in A549 treated with alkanal 10µM, but no effect of JF0064 was detected. B) The AKR Inhibitor JF0064 reduced about 40% of the production of alkanol. A dose-dependant response was observed between tolerestat and the inhibition of alkanol production in H460 treated with alkanal 10µM. C) AKR1B10 knockouts and AKR1B15 knockouts with reduced expression of AKR1B10 show reduced production of alkanol under alkanal administration (10µM). AKR1B15 knockout without AKR1B10 downregulation showed 20% reduction in the production of alkanol compared to wild-type cells similar to the overall reduction in AKR activity detected by colorimetric assay. Data normalized by vehicle/WT group (first timepoint).*

4. Conclusions

We have demonstrated the capacity to detect VOCs from in vitro headspace.

Using *in vitro* study of human lung cancer cells, we have demonstrated the potential to monitor the metabolic conversion of administered EVOC Probe aldehydes into alcohols by AKRs, and have used AKR inhibition/silencing to show that this relationship is specific and sensitive to manipulation.

Pre-clinical samples were analysed using the same GC-MS workflow used for the detection of VOCs in clinical breath samples (OML Breath Biopsy® workflow). We are now seeking to investigate the same relationships through *in vivo* and *ex vivo* sampling to further establish the potential to use EVOC Probes targeting AKR metabolism as a tool for early detection of lung cancer.