Investigating changes in TCA cycle metabolism associated with isocitrate dehydrogenase (IDH) mutations in brain tumours

Khalid Al-Qahtani[1,3] , Chiara Bardella[2], Inga Pfeffer[1],Daniel Krell[2], Ian Tomlinson[2], Christopher J. Schofield and James S. O. McCullagh[1],

[1]Department of Chemistry, University of Oxford, South Parks Road, Oxford UK

[2]Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford UK

[3]Metabolic Screening Laboratory, King Faisal Specialist Hospital and Research Center, Riyadh 11211, Saudi Arabia

OVERVIEW

Mutations in metabolic enzymes, including isocitrate dehydrogenase 1 & 2 (IDH1 & 2) found in brain tumours, strongly implicate altered metabolism in tumourigenesis. IDH1 and IDH2 catalyze the inter-conversion of isocitrate and 2-oxoglutarate (2OG), a TCA cycle intermediate and an essential co-factor for a number of Oxygenase enzymes. Here we present the application of a number of novel LC/MS based methods to identify and quantify TCA cycle intermediates and the 2 enatiomers of 2-Hydroxyglurate in IDH mutant and wild type cell lines.

INTRODUCTION

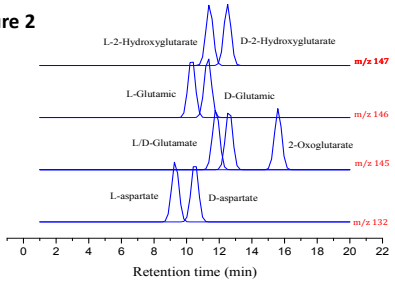
I. IDH1 Mutations in Gliomas: "When an Enzyme Loses Its Grip"

Cancer-associated IDH point mutations alter IDH e enzymes enabling an unusual change of function where 2OG is converted to the structurally similar metabolite 2-hydroxyglutarate, a polar, chiral dicarboxylic acid that exists in two configurations, D-2-HG and L-2-HG. The pathway for this formation can be seen in **Figure 1.** We were interested in investigating whether other TCA cycle intermediates were affected by the mutations of IDH and specifically which enantiomer of 2HG accumulated.

II. Chiral liquid chromatography LC–MS in the determination of the configuration of 2-hydroxyglutaric acid in Cancer

We developed two selective and sensitive LC/MS method for the identification and quantification of intracellular metabolites involved in TCA cycle metabolism. The first separated all TCA cycle intermediate (except oxaloacetate which quickly breaks down in cell extracts) (see Fig4). The second separated D and L forms of 2-hydroxyglutarate, (D & L) glutamine, (D & L) glutamate and (D & L) aspartate. The separation of charged enantiomeric forms is performed using a ristocetin A glycopeptide antibiotic silica gel bonded column. The eight enantiomeric forms of the compounds were well separated with baseline resolution at 11.42/12.41 min for the L/D-2-HG, 11.80/12.11 min for the L/D-glutamic and at 10.31/11.18 for the L/D-glutamine for the elution chromatography see **Figure 2**.

Figure 2



III. Chromatography

The mobile phase used for the enantiomeric separation for 2-HG consisted of a mixture of: (A) 10 mM triethylamine adjusted to pH 7.0 with acetic acid; and (B) acetonitrile with 0.1% formic acid, for the affect off formic acid and acetonitrile rate see **Figure 3**. The column used was a Chirobiotic R column of 250 mm length × 4.6 mm I.D., 5 mm silica gel particles bonded to the macrocyclic glycopeptide ristocetin A (Advanced Separation Technologies, Whippany, NJ, USA). The flow rate was 0.5mL/min with a split where the effluent from the column was connected to a dead-volume T-connection before it reached the mass spectrometer, allowing only 150µL/min to flow into the ion source.

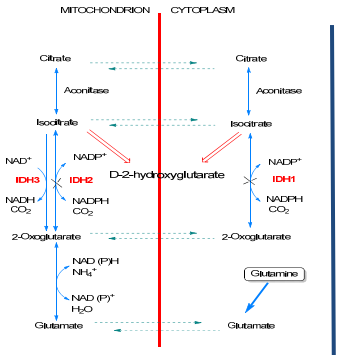
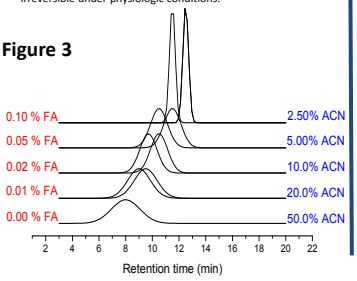


Figure 1

The role of the IDH family of enzymes in the TCA cycle.

The main mitochondrial and cytoplasmic reactions that involve IDH are shown here which reversibly catalyzes the NADP+dependent decarboxylation of isocitrate to 2OG in the cytoplasm. IDH2 mediates the same reaction in the mitochondria. IDH3 catalyzes the NAD+-dependent conversion of isocitrate to 2OG in the mitochondria in a reaction that is irreversible under physiologic conditions.

Figure 3



III. The application of novel LC/MS for the quantifications of TCA cycle intermediates in human gliomas

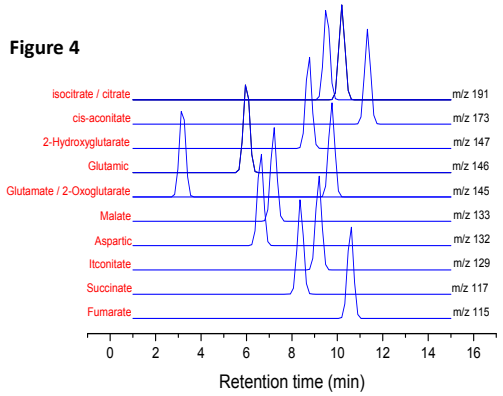
Additionally, we developed and applied a novel liquid chromatography-mass spectrometry method for studying changes in TCA cycle intermediates and their associated metabolite concentrations. To subsequently apply these in cell and in vivo based studies to identifying and quantifying oncometabolites. This method used a hybrid stationary phase with an Acquity HSS T3 column (hydrophilic C18 retention mechanism) Gradient **Table 1**. This provides a unique retention most likely based on hydrophobic and hydrophilic interactions between the stationary phase and the analyte. Under the optimized chromatographic conditions, the two isomers were well separated with isocitrate eluting at ca. 10.20 min followed by the isomer citrate at ca 9.35min , for the UPLC–MS chromatogram see **Figure 4**. The method was linear for 2HG in the range of (2.5 – 1000) µM with a correlation coefficient r2 >0.998. However, of the very wide range of concentration covered we found that we may needed to dilute In case 2HG concentrations exceeded that level, the sample was diluted five - to ten-fold with de-ionized water and re-analyzed.

Table 1

Time (min)	Flow Rate ml/min	% of Mobile phase A	% of Mobile phase B
Initial	0.180	100.00	0.00
8.00	0.180	65.00	35.00
9.00	0.180	100.00	0.00
15.00	0.180	100.00	0.00

These LC–MS/MS methods were developed and applied to the analysis of cancer cell tissues to investigate changes in citric acid cycle intermediates and the identify selected enantionmer concentrations. Here we describe the methodology used and give examples from the analysis of selected wild-type and modified cancer cell lines which show highly specific enatiomeric changes taking place in mutant cell lines.

Figure 4



RESULTS

I. Human cells expressing assay

To understand the impact and integrate of IDH1 and IDH2 mutation on cellular metabolism, we profiled metabolites to identify changes in metabolite levels in cells expressing R132 mutant IDH1 and R172 IDH2 compared with cells expressing wild-type IDH1 and 2. see figure 5

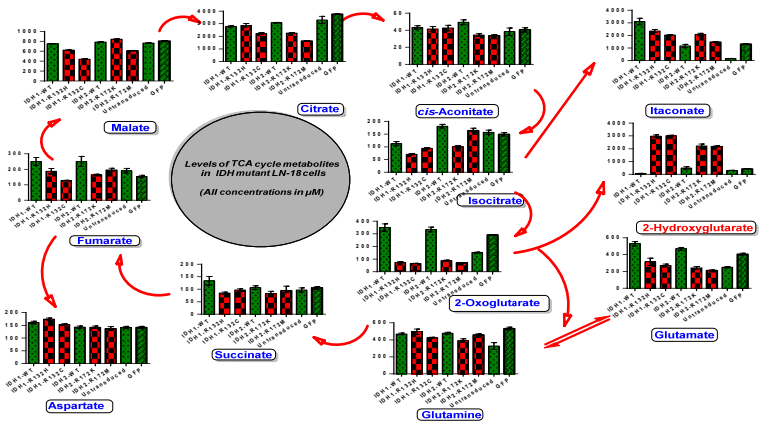


Figure 5 Results showing concentrations of TCA cycle intermediates including 2-Hydroxyglutarate for mutant (red columns) and wild type (green columns) cells lines . Note for the mutants the markedly elevated levels of 2-HG with concomitantly low levels of 2-OG.

II. chiral liquid chromatography Validation

Calibration curves were linear up to at least 2000 µM with r2 value equal to 0.997 and detection limits for (D/L)-2HG, 10µM; 2-OG, 8µM) signal-to-noise ratio(S/N) of around 3:1. Inter-day (n=3) and intra-day coefficient of variation were better than 13.6%, The standard deviation (SD) were around 7.3 and the percentage coefficient of variation (%CV) were low than 3 of all TCA-cycle metabolites the assay was successfully applied to control (n=4) and 2-hydroxyglutaric (n=4) cells expressing samples.

Figure 6

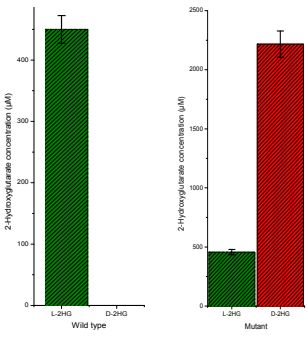
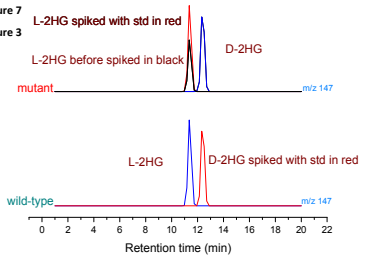


Figure 6. Spectrometric details supporting the identification of species D/L- 2HG are shown in figure 6. Error bars depict one standard deviation (s.d.) from the mean of three independent experiments.

Figure 7

Figure 3



III. Determination of configuration of the Chiral 2-HG in WT and mutant

Peak identification of the two configurations of (D/L)-2-HG was established by analyzing commercially available standards using chromatographic conditions described above. For analysis of mutant IDH1 and R172 IDH2 compared with cells expressing wild-type IDH1 and 2, added for each freeze dried samples (extracted by 80% MeOH with Water from the cells) 50µL mobile phase, vortexes, and filtered through a 0.45 mm membrane filter. The filtrate was injected (5µL) into the mass spectrometer. LC-MS/MS negative ion chromatograms obtained in the SIR mode of analysis using Chirobiotic R column. Wild-type sample from normal cells spiked with standard D-2-HG and cancer cells (mutant) spiked with standard L-2-HG in figure 7 .

SUMMARY

I. Two LC/MS methods were developed :The first to identify and quantify TCA intermediates and the second to resolve a number of enantiomers including D and L forms of 2-Hydroxyglutarate.

I. 2-Oxoglutarate levels are reduced in mutant cell lines compared to wild type

II. Mutant cells expressing R132H IDH1 and R172K IDH2 show significantly elevated levels of D-2HG.

III. Low levels of L-2HG are found in both the wild type and mutant cells. The D-form however is increased significantly in mutants.

References

1. Dang L, White DW, Gross S, Bennett BD, Bittinger MA, Driggers EM, Fantin VR, Jang HG, Jin S, Keenan MC, et al. 2009. Cancer-associated IDH1 mutations produce 2-hydroxygluta-rate. Nature 462: 739–744.
2. Ward PS, Patel J, Wise DR, Abdel-Wahab O, Bennett BD, Coller HA, Cross JR, Fantin VR, Hedvat CV, Perl AE, et al. 2010. The common feature of leukemia-associated IDH1 and IDH2 mutations is a neomorphic enzyme activity converting a-ketoglutarate to 2-hydroxyglutarate. Cancer Cell 17: 225–234
3. Reitman ZJ, Yan H. 2010. Isocitrate dehydrogenase 1 and 2 mutations in cancer: Alterations at a crossroads of cellular metabolism. J Natl Cancer Inst 102: 932–941.