

# Autophagy induced by DCA treatment, PI3K inhibition or starvation results in reduced pyruvate to lactate exchange observed by DNP <sup>13</sup>C-MRS.

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**INTRODUCTION:** Autophagy is a cellular degradation response to starvation or stress whereby cellular proteins, organelles and cytoplasm are engulfed, digested and recycled to sustain cellular metabolism. This process could paradoxically allow cancer cells to survive in hostile environments. However, persistent autophagy causes cells to become depleted in organelles and critical proteins which ultimately lead to cell death [1]. The TCA cycle is predicted to become more active during autophagy, as the amino acids and fatty acids generated by the autophagic process are utilised to produce energy in the TCA cycle. Currently, there is no non-invasive method to monitor the autophagic processes and autophagy.

**AIM:** To investigate the effects of autophagy on the activation of the TCA cycle in cells by dynamic nuclear polarisation (DNP) and <sup>13</sup>C-MRS, in order to develop a non-invasive marker for autophagy.

**METHODS:** Bax deficient colon carcinoma HCT116 cells (HCT116 Bax-ko) were used to induce autophagy through either 6 or 24 hours of starvation (HBSS media) or 24 hours exposure to 10µM PI-103 (a PI3K inhibitor) treatment. Bax deficiency prohibits the cells ability to undergo apoptosis, resulting in the induction of autophagy during cellular stress. HCT116 Bax-ko cells were also treated with the pyruvate dehydrogenase kinase inhibitor, dichloroacetate (DCA, 75mM) for 24 hours, in order to study the effect of TCA cycle activation [2] on the rate of pyruvate to lactate exchange and compare it with the effect of autophagy on TCA cycle activation. Electron microscopy, Annexin V/propidium iodide (PI) flow cytometry analysis and western blotting provided markers of autophagy and apoptosis. The hyperpolarised [<sup>1-<sup>13</sup>C</sup>] pyruvate assay by DNP and <sup>13</sup>C-MRS was used to non-invasively monitor the rate of [<sup>1-<sup>13</sup>C</sup>] labelled pyruvate to lactate exchange in real time following the induction of autophagy and in response to DCA. Rates were derived from non-linear least squares fitting of the bi-exponential time dependence of the hyperpolarised lactate and pyruvate signals. Culture media and extracts from the treated and control cells were analysed by <sup>1</sup>H-MRS. Lactate dehydrogenase (LDH) expression and activity were also examined. Surprisingly, DCA was found to induce autophagy in HCT116 Bax-ko cells. In order to confirm this effect was not only limited to this cell line, the response of HT29 (colon carcinoma) and PC3 (prostate carcinoma) cells to DCA was similarly investigated.

**RESULTS:** Molecular markers of autophagy by western blots (increased LC3II expression) and electron microscopy (presence of double-membrane autophagosomes) confirmed the induction of autophagy in HCT116 Bax-ko cells by starvation, DCA or PI103 treatment and in DCA treated PC3 and HT29 cells (Fig.1 and 2). All treatment groups were found to exhibit minimal apoptosis or necrosis by Annexin V/PI flow cytometry analysis and western blots (absence of cleaved PARP and cleaved Caspase-3, Fig 1). Significant reductions in the rate of hyperpolarised labelled [<sup>1-<sup>13</sup>C</sup>] pyruvate to lactate exchange measured in real-time by DNP <sup>13</sup>C-MRS were found in all treatment groups, except for the 6 hours starvation group (Fig. 3). <sup>1</sup>H-MRS of the culture media from all the 24 hours treatment groups also showed a reduction in steady state unpolarised lactate production. No significant change in LDH expression or activity or in the concentration of its co-factor NAD<sup>+</sup> was found in any treatment group.

**DISCUSSION:** Autophagy was induced in three cancer cell lines by three different treatment regimes. To our knowledge, this is the first study to show DCA induced autophagy in cancer cells. The exchange of the hyperpolarised [<sup>1-<sup>13</sup>C</sup>] label in pyruvate to lactate has been previously used to study the activity of LDH and its response to treatment in cancer [3, 4], and shown to decrease following drug induced cell death, attributed to apoptosis [4]. The observed significant reductions in the rate of labelled [<sup>1-<sup>13</sup>C</sup>] pyruvate to lactate exchange (and unpolarised lactate production) herein was associated with autophagy, rather than apoptosis or necrosis. The decrease in lactate production measured in real time, together with unchanged cellular NAD<sup>+</sup> and LDH expression and activity could be attributed to: i) more pyruvate being diverted to the TCA cycle, as the TCA cycle becomes more active during autophagy; and/or ii) LDH being sequestered in autophagic vacuoles [5]. The rate of labelled [<sup>1-<sup>13</sup>C</sup>] pyruvate to lactate exchange remained unchanged in HCT116 Bax-ko cells after 6 hours of starvation, perhaps because the autophagic vacuoles were not yet fully formed and the cellular proteins, organelles and cytoplasm are not yet engulfed and digested, hence the TCA cycle is not yet activated and/or the LDH is not yet being sequestered at this time point.

**CONCLUSIONS:** A reduction in lactate production as measured by DNP <sup>13</sup>C-MRS, and unchanged NAD<sup>+</sup>, may provide a non-invasive means of assessing autophagy.

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1) Mathew *et al.*, *Nat Rev Cancer* (2007). 2) Bonnet *et al.*, *Cancer Cell* (2007). 3) Ward, *et al.*, *Cancer Res* (2010). 4) Day *et al.*, *Nature Med* (2007). 5) Houri *et al.*, *Biochem J* (1995).

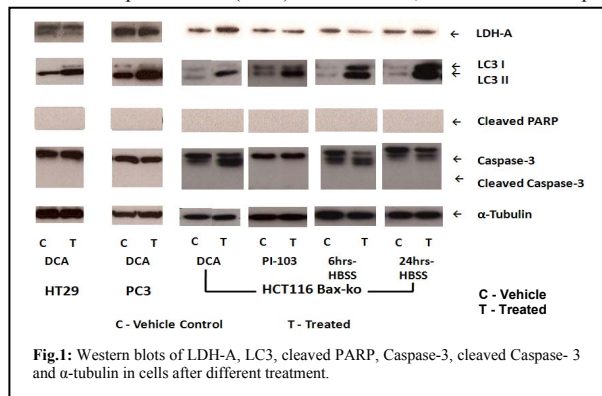


Fig.1: Western blots of LDH-A, LC3, cleaved PARP, Caspase-3, cleaved Caspase-3 and α-tubulin in cells after different treatment.

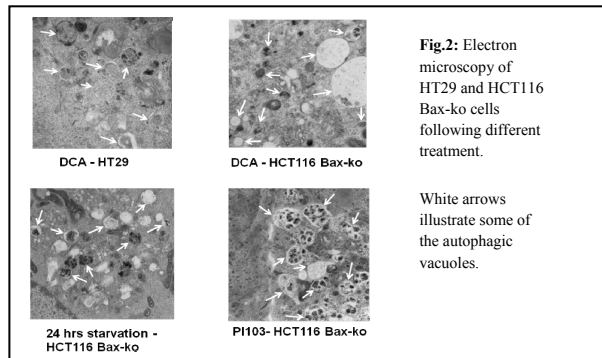


Fig.2: Electron microscopy of HT29 and HCT116 Bax-ko cells following different treatment.

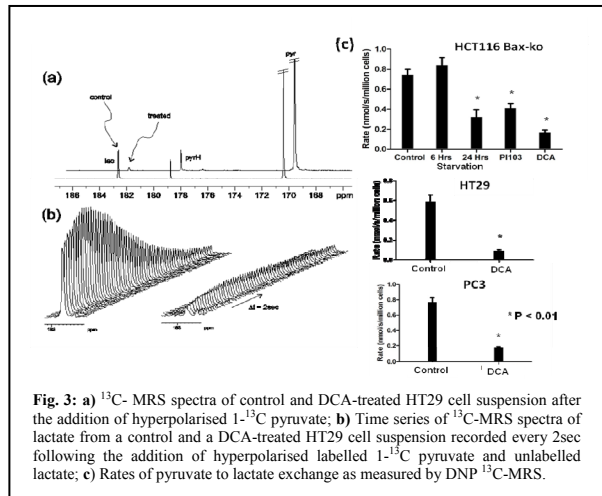


Fig. 3: a) <sup>13</sup>C-MRS spectra of control and DCA-treated HT29 cell suspension after the addition of hyperpolarised <sup>1-<sup>13</sup>C</sup> pyruvate; b) Time series of <sup>13</sup>C-MRS spectra of lactate from a control and a DCA-treated HT29 cell suspension recorded every 2sec following the addition of hyperpolarised labelled <sup>1-<sup>13</sup>C</sup> pyruvate and unlabelled lactate; c) Rates of pyruvate to lactate exchange as measured by DNP <sup>13</sup>C-MRS.