Effect of the HIF Pathway Inhibitor NSC-134754 on Glucose Metabolism

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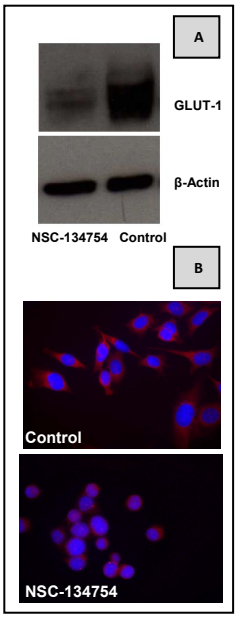
Introduction:
Hypoxia within tumours impacts negatively on both chemo- and radiotherapy [1]. The master regulator in hypoxic cell survival and adaptation is the hypoxia-inducible factor-1 (HIF-1) pathway [2]. Glucose transporter 1 (GLUT-1) is an important downstream target of the HIF-1 pathway, with fundamental roles in intracellular glucose flux and availability. We have previously shown the anti-tumour activity of the HIF pathway inhibitor NSC-134754 through a significant increase in tumour ADC *in vivo* [3]. Given the intrinsic role of GLUT-1 in glucose metabolism (and cellular bioenergetics), the aim of our study was to determine whether GLUT-1 suppression with NSC-134754 evoked a unique metabolic response stimulated under hypoxic conditions. We have examined the metabolic effects of NSC-134754 *in vitro* using magnetic resonance spectroscopy (MRS), with validation using western blotting and immunocytofluorescence. Furthermore, we have confirmed the effect of NSC-134754 on glucose transport *in vitro* extends to an *in vivo* orthotopic prostate model using immunohistochemistry.

Methods:
Human PC3LN5 prostate tumour cells were cultured in T75cm^3 flasks, and on 6 well plates +/- 20 x 20mm coverslips. Cells were maintained at 37ºC in a humidified incubator with an atmosphere of 5% CO_2 in 95% air. NSC-134754 was obtained from the National Cancer Institute's Developmental Therapeutics Program. At semi-confluence, medium was removed and 7.5µM NSC-134754 supplemented medium was added 30 minutes before incubation in a humidified hypoxic workstation pre equilibrated to 1.0% O_2, 5% CO_2 at 37˚C. After 24h, culture media samples were collected from the flasks and metabolites extracted using the standard dual phase cell extraction method. Water-soluble metabolites were lyophilised, re-suspended in D_2O and neutralised, with TSP (3-trimethylsilyl-[2,2,3,3-2H4]-propionic acid) added as an internal reference standard. High resolution ^{1}H NMR spectroscopy was performed on conditioned media samples and cell extracts using a 500MHz Bruker MR system. Cells cultured in 6 well plates were analysed for GLUT-1 expression by western blotting and GLUT-1 membrane/cellular distribution by immunocytofluoresecence. For *in vivo* studies, tumours were propagated by injecting 1x10^5 PC3LN5 cells orthotopically into the ventral prostate gland of male NCr nude mice. When approximately 1cm in diameter (measured by palpation) mice were given 100mg/kg i.p. NSC-134754 (n=4) or vehicle alone (n=5). After 24h, tumours were excised, fixed in 10% formalin and embedded in paraffin. GLUT-1 immunohistochemistry was performed on 5µM sections and analysed using a semi-quantitative scoring system [4].

Results:
Western blot analysis demonstrated a reduction in GLUT-1 expression in NSC-134754 treated cells compared to controls after 24h in hypoxia (1% O_2) (Fig.1A). Immunocytofluorescence confirmed hypoxic control cells exhibited extensive membranous staining with diffuse cytoplasmic expression of GLUT-1. In cells treated with NSC-134754, a decrease in both GLUT-1 expression and distribution was evident (Fig. 1B). ^{1}H MRS analysis of conditioned media from NSC-134754 treated cells showed a statistically significant decrease in both glucose consumption and lactate production compared to control cells. Intracellular lactate concentration was not significantly different between groups. However, treatment with NSC-134754 significantly increased intracellular glucose by >3 fold compared to controls (Fig. 1C). Other metabolites of note were glutamine and glutamate, both of which were: i) significantly increased in uptake from conditioned media and ii) had significantly increased intracellular levels ($p<0.05$, data not shown) in NSC-134754 treated cells compared to controls. Immunohistochemical analysis of PC3LN5 orthotopic prostate tumours demonstrated a 28±2% decrease in GLUT-1 membrane expression in treated tumours at 24h compared to controls ($p<0.01$, Kruskal-wallis test) (Fig. 1D).



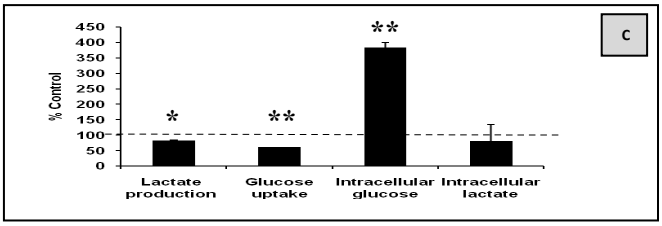
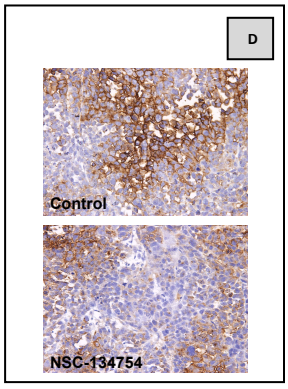


Figure 1. (**A-C**) Treatment of PC3LN5 cells with 7.5µM NSC-134754 for 24h in hypoxia; effects on glucose transport, glucose metabolism and lactate levels. **A)** Western blot showing GLUT-1 expression. **B)** Immunocytofluorescence of GLUT-1 expression and distribution (red) with dapi stained nuclei (blue) x400. **C)** ^{1}H MRS-detected metabolite changes with NSC-134754. *$p<0.05$, **$p<0.01$, Student's t-test). **D)** Representative images of *ex vivo* GLUT-1 staining of PC3LN5 orthotopic prostate tumours, x200.



Conclusions:
The results of this study demonstrate that treatment with the HIF pathway inhibitor NSC-134754 had significant effects on the metabolic profile of PC3LN5 cells *in vitro*. After 24h in hypoxia, treated cells downregulated the expression and distribution of GLUT-1, with a concurrent decrease in glucose uptake. Our findings also show that treatment with NSC-134754 is associated with a reduction in lactate production, which may be the result of decreased anaerobic glucose consumption. Both a reduction in glucose transport and lactate production have been shown to be indicators of response to therapy [5, 6]. *Ex vivo* immunohistochemical analysis confirmed a decrease in GLUT-1 is sustained *in vivo* for at least 24h after administration of a single dose of 100mg/kg NSC-134754, suggesting the actions of this compound *in vitro* may provide an indication of the translatable effects of this compound *in vivo*. Interestingly, we have also shown that treatment with NSC-134754 *in vitro* increased intracellular glucose accumulation. As many glycolytic enzymes are known to be gene targets of HIF, a reduction in their availability (and therefore glycolysis) could lead to glucose trapping. To our knowledge, this is a novel finding of the metabolic actions of a small molecule inhibitor of the HIF pathway. Increased intracellular glucose coupled with decreased glucose uptake has been reported as an early response to other therapeutics *in vitro* [7]. The implications of this effect in the context of HIF inhibition *in vivo* need to be established. Finally, the increased metabolism of glutamine/glutamate induced by NSC-134754 *in vitro* could be a mechanism to compensate for the reduction in glucose transport and metabolism in order to sustain cellular bioenergetics. In conclusion, HIF inhibitors are an emerging class of therapeutics, whose precise mechanism of action is unclear. We therefore propose the use of MRS to evaluate the effects of novel HIF inhibitor agents, which may exhibit unique metabolic fingerprints.

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