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Inhibition of endogenous hydrogen sulfide production in clear-cell renal cell carcinoma cell lines and xenografts restricts their growth, survival and angiogenic potential

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ABSTRACT

Clear cell renal cell carcinoma (ccRCC) is characterized by Von Hippel–Lindau (VHL)-deficiency, resulting in *pseudohypoxic*, angiogenic and glycolytic tumours. Hydrogen sulfide (H_2S) is an endogenously-produced gasotransmitter that accumulates under hypoxia and has been shown to be pro-angiogenic and cytoprotective in cancer. It was hypothesized that H_2S levels are elevated in VHL-deficient ccRCC, contributing to survival, metabolism and angiogenesis. Using the H_2S-specific probe MeRhoAz, it was found that H_2S levels were higher in VHL-deficient ccRCC cell lines compared to cells with wild-type VHL. Inhibition of H_2S-producing enzymes could reduce the proliferation, metabolism and survival of ccRCC cell lines, as determined by live-cell imaging, XTT/ATP assay, and flow cytometry respectively. Using the chorioallantoic membrane angiogenesis model, it was found that systemic inhibition of endogenous H_2S production was able to decrease vascularization of VHL-deficient ccRCC xenografts. Endogenous H_2S production is an attractive new target in ccRCC due to its involvement in multiple aspects of disease.

Abbreviations: RCC, renal cell carcinoma; mRCC, metastatic renal cell carcinoma; ccRCC, clear cell renal cell carcinoma; VHL, Von Hippel–Lindau; HIF, hypoxia-inducible factor; VEGF, vascular endothelial growth factor; mTOR, mammalian target of rapamycin; H_2S, hydrogen sulfide; CSE, cystathionine γ-lyase; CBS, cystathionine β-synthase; MPST, 3-mercaptopyruvate sulfurtransferase; ETC, electron transport chain; SQR, sulfide quinone reductase; HA, hydroxylamine; PAG, propargyl glycine; LC, L-cysteine; EGFP, extreme green fluorescent protein; CAM, chorioallantoic membrane; rhodamine-LCA, rhodamine-conjugated *lens culinaris agglutinin*.

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1. Introduction

Renal cell carcinoma (RCC) has a cancer-specific mortality rate of 30–40% [1]. Despite advances in early detection of RCC, upto 30% of RCC patients present with metastatic disease (mRCC), which is highly resistant to systemic chemotherapy and radiation therapy [2]. This has led to the development of alternate RCC therapies that target the molecular basis of the cancer [3].

Of the various histological subtypes of RCC, the most common is the clear cell histotype (ccRCC), accounting for roughly 80% of all RCCs [4]. A common molecular signature of ccRCC, present in 90% of cases, is inactivation of the Von Hippel–Lindau (VHL) tumour suppressor, a protein responsible for the degradation of hypoxia-inducible factors alpha subunits (HIF-1/2α) under normoxia [3,5]. When VHL is inactivated, HIF-1/2α transcription factors are not degraded under normoxic conditions and cells become *pseudohypoxic*, inappropriately upregulating growth factors and pro-angiogenic factors like vascular endothelial growth factor (VEGF) [3]. As such, recently developed ccRCC therapies aim to disrupt these growth and angiogenic signalling pathways by targeting mammalian target of rapamycin (mTOR) – a master regulator of growth and survival – and VEGF receptors on endothelial cells [3].

Unfortunately, objective response rates of these targeted therapies only approach 50% and durable complete responses are rare [1,6]. There is a need for a truly unique targeted therapy for treatment of mRCC, seeing as significant investment into VEGF and mTOR inhibitors has yet to yield a cure for the disease [7]. Another identifying feature of pseudohypoxic ccRCC tumours which may be a promising target is a strong preference for glycolysis over mitochondrial respiration, even when oxygen is readily available [8]. This shift in metabolism is known as the Warburg Effect, and is thought to provide cancer cells with a cellular environment that facilitates rapid proliferation [9]. Rather than targeting cell proliferation, angiogenesis, and metabolism individually, it might be more favourable to target these processes simultaneously. However, finding good molecular targets that mediate multiple aspects of disease has proven challenging.

Hydrogen sulfide (H_2S)'s role as a physiological molecule with pleiotropic functions is becoming increasingly apparent. It is generated endogenously in mammalian cells by three independent enzymes: cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS) and 3-mercaptopyruvate sulfurtransferase (MPST) [10]. Expression of these enzymes within renal epithelial cells is physiologically important because of the role that they play in the detoxification of homocysteine [10,11]. Production of H_2S was long regarded as simply a by-product of this process, however there is now evidence that this small gaseous molecule serves diverse functions in the kidneys and throughout the body [12,13]. In the vascular system, H_2S functions as a potent vasodilator and pro-angiogenic factor that works in combination with nitric oxide and VEGF [14–16]. Throughout the body H_2S has been shown to be cytoprotective and antioxidative in various models of injury and ageing [17,18], and has also been shown to be mitogenic [19].

Under normoxic conditions, H_2S is rapidly oxidized by mitochondria through the combined action of the electron transport chain (ETC), the enzyme sulfide quinone reductase (SQR), and the sulfide-oxidizing unit [20–22]. Aerobic mitochondrial oxidation of H_2S not only ensures that H_2S does not reach a toxic concentration at which Complex IV of the ETC is inhibited, but electrons derived from this oxidation also help to drive mitochondrial ATP production [22]. However under hypoxic conditions when oxygen is scarce, mitochondrial oxidation of H_2S ceases, allowing H_2S to accumulate and function as an oxygen sensor [23,24]. H_2S accumulation under hypoxia helps maintain cell function by upregulating anaerobic metabolic pathways like glycolysis [25], upregulating cytoprotective pathways [26], and helping to restore oxygen supply [15,27].

Given its involvement in angiogenesis, cytoprotection and metabolism, increased H_2S production has been linked to various cancers. It has recently been shown that upregulation of CBS in colon cancer cells produces H_2S at a level that is capable of sustaining the bioenergetics of malignant colonocytes, which in turn leads to increased proliferation [19,23,28]. In addition, CBS upregulation in tumour cells increases tumour neovascularization [28]. H_2S production has also been linked to play a role in cancers of the ovaries, breast and the liver where it may play a role in conferring resistance to radiation therapy, chemotherapy and hypoxia by maintaining supply of glycolytic and antioxidative substrates [29–32]. While the expression of H_2S-producing enzymes has not been investigated in ccRCC nor other renal cancers, it was recently found that the expression of CBS is regulated in part by HIF-1/2α subunits [33]. This leads us to hypothesize that expression of endogenous H_2S-producing enzymes might be increased in psuedohypoxic ccRCC cell lines, and may contribute to the survival, metabolism and angiogenesis of ccRCC tumours.

Here we describe for the first time that endogenous levels of H_2S are increased in VHL-deficient ccRCC cell lines and inhibition of H_2S-producing enzymes can significantly decrease the growth, survival, metabolic output and angiogenic potential of ccRCC cell lines. The cell-permeable, H_2S-specific, fluorescent probe MeRhoAz was used to measure intracellular levels of H_2S in ccRCC cell lines at baseline, and following inhibition/stimulation of H_2S-producing enzymes. We evaluated the effects of inhibiting/stimulating endogenous H_2S production on ccRCC cell lines *in vitro* by quantifying cell growth, metabolism and viability in the VHL-deficient ccRCC cell lines 786-O and 769-P, as well as the VHL wild-type ccRCC cell lines Caki-1 and the 786-O VHL knock-in (786-O VHL+). We further evaluated the effects of H_2S inhibition on xenograft neovascularization *in vivo* using an avian xenograft model previously developed for RCC [34].

2. Methods

2.1. Cell culture

All cells were cultured under normal growth conditions (37 °C, 5% CO_2, 21% O_2) in growth media (Gibco®) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Subculturing of cells was performed weekly with use of 0.05% trypsin EDTA (Gibco®). The human renal epithelial cell line HK-2 was generously donated by Dr. Lakshman Gunaratnam (Western University, London, ON) and cultured in Keratinocyte Serum Free Medium supplemented with human recombinant Epidermal Growth Factor 1–53 and Bovine Pituitary Extract. The VHL wild-type ccRCC cell line Caki-1 was generously donated by Dr. Alison Allan (Western University, London, ON) and cultured in McCoy's 5A growth medium. The VHL-deficient ccRCC cell lines 769-P and 786-O were also donated by Dr. L. Gunaratnam and cultured in Dulbecco's Modified Eagle Medium. The ccRCC 786-O VHL+ cell line was generously donated by Dr. James Brugarolas (UT Southwestern, Dallas, TX) and grown in Dulbecco's Modified Eagle Medium.

2.2. Treatments

Inhibitors of endogenous H_2S synthesis – hydroxylamine (HA) and propargyl glycine (PAG) – and the substrate for endogenous H_2S production – L-cysteine (LC) – were prepared as 100 mM stock solutions in PBS. Effective doses ranged from 0.5 mM to 5 mM, depending on the assay, and were used to treat cells seeded in 96-well, 24-well, 12-well or 6-well plates. Cells were treated for