METABOLIC PLASTICITY IN CANCER CELLS: INVOLVEMENT IN THE PROCESSES OF PROLIFERATION AND RESPONSE TO RADIOTHERAPY

Cover figures:
Human cervix cancer cells in culture, optical microscope and transmission electron microscopy of a mitochondrion.

Mes années de doctorat, ce sont quatre années et des poussières de recherches, mais ce sont surtout des rencontres, de précieuses collaborations et de l'aide.

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Abbreviation list

^{18}F-FDG	^{18}F fluorodeoxyglucose
^{18}F-FLT	^{18}F fluorothymidine
2-DG	2-deoxy-D-glucose
2-DG-6-P	2-deoxy-D-glucose-6-phosphate
6-AN	6-aminonicotinamide
6-PG	6-phosphogluconate
ADP	adenosine diphosphate
AMPK	adenosine monophosphate-activated protein kinase
AOA	aminooxyacetate
ATP	adenosine triphosphate
CA	carbonic anhydrase
CBS	cystathionine-β-synthase
CO	carbon monoxide
Cox	cytochrome C oxidase
CSE	cystathionine-γ-lyase
DCA	dichloroacetate
DNA	deoxyribonucleic acid
ENO	enolase
eNOS	endothelial nitric oxide synthase
EPR	electron paramagnetic resonance
ETC	electron transport chain
$FADH_2$	reduced flavin adenine dinucleotide
G-6-P	glucose-6-phosphate
G6PD	glucose-6-phosphate dehydrogenase
GLUT	glucose transporter
GSH	glutathione
GSTZ1	glutathione-S-transferase zeta 1
H_2S	hydrogen sulfide
HIF-1	hypoxia-induced factor 1
HK	hexokinase
K_{ATP}	ATP-sensitive potassium channel
K_i	inhibition constant
K_m	michaelis constant
LDH	lactate dehydrogenase
MCA	microbubble ultrasound contrast agent
MCT	monocarboxylate transporter
mmHg	millimeter of mercury
MPC	mitochondrial pyruvate carrier
Na_2S	sodium sulfide
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NaHS	sodium hydrosulfide
NHE1	sodium-proton exchanger 1

NMDA	N-methyl-D-aspartate
NMR	nuclear magnetic resonance
NO	nitric oxide
OAA	oxaloacetate
OXPHOS	oxidative phosphorylation
PDH	pyruvate dehydrogenase
PDK	pyruvate dehydrogenase kinase
PEP	phosphoenolpyruvate
PET	positon emission tomography
PFK	phosphofructokinase
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3
PGM	phosphoglycerate mutase
pHe	extracellular pH
pHi	intracellular pH
Pi	inorganic phosphate
PK	pyruvate kinase
PKM2	pyruvate kinase isoform 2
pO_2	oxygen partial pressure
ppm	part per million
PPP	pentose phosphate pathway
R-5-P	ribose-5-phosphate
ROS	reactive oxygen species
RP-HPLC	reversed-phase high performance liquid chromatography
RT	radiotherapy
shRNA	small hairpin ribonucleic acid
siRNA	small interfering ribonucleic acid
SMCT	sodium-coupled monocarboxylate transporter
SOU	sulfide oxidation unit
SRB	sulfate reducing bacteria
SUR	sulfonylurea receptor
TCA	tricarboxylic acid
V-ATPase	membrane-bound vacuolar ATPase
VDAC	voltage-dependent anion channel

Foreword

Malignant and non-malignant cells can significantly differ in their way to metabolize glucose. While normal differentiated cells tend to fully oxidize glucose by the coupling of glycolysis and oxidative phosphorylation, some cancer cells adopt glycolysis as a main mode of energy production even in the presence of oxygen. Intense research has led to a globally accepted hypothesis, considering that aerobic glycolysis (also termed the “Warburg Effect”) is beneficial for energy production and proliferation. It has already attracted major interest as it is hoped that drugs targeting glycolysis will be effective in decreasing cancer progression. Demystifying mechanisms by which cancer cells switch to a constitutive glycolytic phenotype is of paramount importance in order to develop efficient therapeutic strategies. It also allows understanding resistance phenomena occurring in some cancer patients. Pioneering studies on cancer metabolism suggested that enhanced glycolysis was caused by irreversible impairment in mitochondrial respiration. Since then, this hypothesis has been repeatedly refuted to some exceptions; and functional mitochondrial activity was reported in cancer cells.

To date, clinical studies on metabolism-targeting drugs have provided encouraging data; but efforts in increasing treatment efficacy, reducing side-effects and avoiding resistance are still needed. In this regard, combination of treatments with other modalities is an important avenue for future clinical development. Hypoxia is particularly considered as a tumor feature to overcome, as it induces resistance to chemo- and radiotherapy. For this reason, molecules that alter oxygen consumption by cancer cells and/or increase their supply of oxygen can be advantageous.

In **chapter 1**, several concepts of cancer metabolism are described and recent advances in drug development are highlighted. Current strategies that tackle glycolysis are first depicted, with a particular focus on the recently discovered molecule dichloroacetate (DCA). Then, the

role of mitochondria in supporting tumor growth is detailed and drugs inhibiting mitochondrial oxidative metabolism are considered. Emphasis is given to approaches consisting in reducing hypoxia at the time of irradiation by decreasing cellular oxygen consumption and/or increasing oxygen supply to cancer cells. The end of this chapter is dedicated to the gaseous molecule hydrogen sulfide (H_2S). It has recently been identified in mammals where it has been found to be implicated in various physiological and pathological processes, including cancer. We give highlights on how H_2S interacts with mitochondria, known as its primary target, and discuss its possible role in cancer.

In **chapter 2**, specific aims of the thesis are described.

Chapter 3 reports findings on the mechanisms by which enhanced glucose metabolization through glycolysis favors cancer cells proliferation. Effects of metabolic modulations are described and new insights on the efficacy of DCA are presented.

In **chapter 4**, the effects of the H_2S donor NaHS in cancer therapy are investigated. Two approaches are considered: NaHS in association with radiotherapy or in monotherapy.

Chapter 5 summarizes and discusses the principal findings. This section also presents future directions and the potential impact on clinical development of metabolism-targeting strategies.

Chapter 1 Introduction

I. Metabolism of cancer cells - The Warburg effect

Deregulation of metabolic pathways is emerging as an important hallmark of cancer *(Hanahan and Weinberg, 2011)*. It was first described by Warburg in the early 20th century that some tumor cells exhibit high levels of glycolysis despite the presence of abundant oxygen (O_2), leading Warburg to propose the hypothesis according to which cancer arises from impaired mitochondrial metabolism *(Warburg, 1956)*. Since then, the Warburg hypothesis on the origin of cancer has largely proven incorrect, with the demonstration that mitochondria most generally have functional oxidative activity in tumor cells *(Nakashima et al., 1984; Zu and Guppy, 2004)*. Nevertheless, constitutive upregulation of glycolysis is a common feature of many tumor types, so that it is now exploited as a diagnostic tool and a promising approach to treat malignant lesions *(Gatenby and Gillies, 2004)*.

1. Definition

In normal differentiated cells, one molecule of glucose is converted to two molecules of pyruvate by a sequence of ten enzymatic reactions that take place in the cytosol. In addition to the generation of pyruvate, glycolysis produces two molecules of ATP, two molecules of water, two molecules of reduced nicotinamide adenine dinucleotide (NADH) and two protons (H^+). In the presence of O_2, glycolysis is coupled to the tricarboxylic acid (TCA) cycle where pyruvate is oxidatively metabolized to CO_2; producing additional NADH molecules, H^+, guanosine triphosphate (GTP) and reduced flavin adenine dinucleotide ($FADH_2$). Reductive equivalents produced by glycolysis (that enter into mitochondria *via* the malate-aspartate shuttle) and by the TCA cycle serve as electron donors to the mitochondrial electron transport chain (ETC). By passing through complexes I to IV, transported electrons create a gradient of protons across the inner mitochondrial membrane. They ultimately react with O_2 to produce water. When the protons that accumulate in the intermembrane space are

transferred back to the mitochondrial matrix by ATP synthase, up to 36 molecules of ATP are produced per molecule of glucose, a process known as oxidative phosphorylation (OXPHOS) *(Rolfe and Brown, 1997)*. When the O_2 supply is limited, pyruvate is converted to lactate by the lactate dehydrogenase (LDH) enzyme rather than being used in the TCA cycle and glycolysis becomes the primary source for ATP production (anaerobic glycolysis) *(Rolfe and Brown, 1997)* (figure 1). Compared to normal differentiated cells, some cancer cells can consume glucose at a higher rate and convert pyruvate to lactate even in the presence of O_2 (aerobic glycolysis or the "Warburg Effect") *(Vander Heiden et al., 2009)*. It has however been demonstrated that some proliferating normal cells, such as stem cells *(Folmes et al., 2011)* and activated immune cells *(Hedeskov, 1968)*, can also exhibit a highly glycolytic metabolism (figure 1).

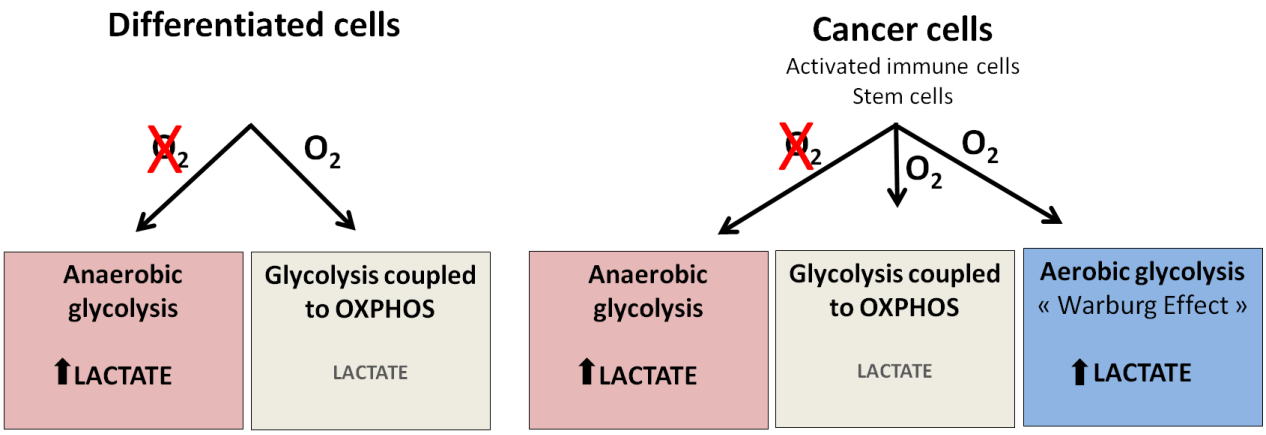

Figure 1: In the presence of oxygen, normal differentiated cells couple glycolysis to oxidative phosphorylation for optimal ATP synthesis. Compared to normal differentiated cells, some cancer cells exhibit a constitutive upregulation of glycolysis even in the presence of an adequate oxygen supply (aerobic glycolysis or the "Warburg Effect"). This particular phenotype is characterized by elevated glucose consumption and lactate production and is also found in some rapidly dividing normal cells.

The Warburg Effect counteracts the Pasteur Effect that operates in most mammalian cells and finely tunes cell metabolism in function of the local O_2 concentration *(Wu and Racker, 1959)*. In normoxia, key glycolytic enzymes of normal cells (hexokinase (HK) -1, phosphofructokinase (PFK) -1, pyruvate kinase (PK) -1) are allosterically repressed by energetic metabolites (ATP, citrate, glucose-6-phosphate). Conversely, when the O_2 supply is compromised, the glycolytic flux is accelerated because of the decreased levels of ATP and citrate produced oxidatively *(Porporato et al., 2011)*. To keep an elevated glycolytic flux, Warburg-phenotype cancer cells develop mechanisms to escape the Pasteur Effect. This can be achieved by actively consuming ATP in anabolic reactions *(DeBerardinis et al., 2008)*. Fang and colleagues also proposed an elegant manner by which cancer cells promote protein glycosylation in the endoplasmic reticulum, which elevates ATP consumption and derepresses the activity of the rate-limiting enzyme PFK *(Fang et al., 2010)*. Finally, a specific isoform of HK (HKII) that has no allosteric site can be overexpressed in cancer cells *(Shinohara et al., 1994; Pedersen et al., 2002)*.

Switching to aerobic glycolysis is the most popular metabolic remodeling described in tumor cells. However, bioenergetic studies of the last decade have highlighted that not all tumor cells rely on intense glycolysis. Different cancer types and different populations of cancer cells inside a given tumor can indeed show variable reliance on glycolysis and OXPHOS for ATP production *(Jose et al., 2011; Moreno-Sanchez et al., 2014)*. Metabolic activities may indeed be affected by the availability of O_2 and nutrients. Some tumor areas may be subjected to O_2 and/or nutrient deprivation caused by insufficient perfusion *(Vaupel et al., 1989)*, thereby leading to cell death or prompting cancer cells to use alternative metabolic routes. As an example, preservation of an oxidative metabolism by oxygenated tumor cells can be an advantageous manner to spare glucose for glycolytic tumor cells *(Sonveaux et al., 2008)*. In the following sections, emphasis on the role of aerobic glycolysis

in tumor biology is first given. Then, evidence and importance of functional mitochondrial oxidative activity in tumor cells is discussed.

2. Molecular mechanisms of aerobic glycolysis

Mutations or epigenetic changes mainly occurring in the phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR, hypoxia-inducible factor 1 (HIF-1) and Myc signal transduction systems participate in various facets of the Warburg Effect, as reviewed in *(DeBerardinis et al., 2008; Lunt and Vander Heiden, 2011)*.

Metabolic effects of activation of the PI3K/Akt/mTOR pathway include an increased uptake of glucose caused by an increased expression of glucose transporters *(Wieman et al., 2007)*, and an enhanced glycolytic flux through the modulation of expression and activity of glycolytic enzymes *(Deprez et al., 1997; Elstrom et al., 2004)*. Supporting that this pathway is a major driver of aerobic glycolysis in cancer cells, activation of Akt in non-transformed and tumor cells is sufficient to induce a Warburg Effect *(Plas et al., 2001; Rathmell et al., 2003)*. Moreover, mutations that activate PI3K or its effectors constitute the most prevalent class of mutations in human tumors *(DeBerardinis et al., 2008)*.

HIF-1 is a heterodimer composed of HIF-1α and HIF-1β subunits, and the biological activity of HIF-1 is determined by the stability of HIF-1α, whose level of expression is regulated by O_2 levels *(Kaelin and Ratcliffe, 2008)*. Regulation of HIF-1α expression is posttranslational: in oxygenated cells, proline hydroxylation promotes HIF-1α association with the von Hippel-Lindau (VHL) protein complex, targeting HIF-1α for poly-ubiquitylation followed by destruction by the proteasome. Oxygen is a limiting substrate for the proline hydroxylation of HIF-1α. Therefore, during hypoxia, HIF-1α escapes proteolytic degradation to migrate into the cell nucleus where it binds to HIF-1β. In tumors, constitutive stabilization of HIF-1α can occur *(Isaacs et al., 2005; Selak et al., 2005; Pollard et al., 2007)*. This leads to the increased expression of glucose transporters and glycolytic enzymes, as well as the promotion of pyruvate conversion to lactate *(Semenza, 2010)*.

Finally, alterations in the function of oncogenes like Myc *(Osthus et al., 2000; Ahuja et al., 2010)* and Ras *(Yun et al., 2009)* or of tumor suppressor genes like *p53 (Cheung and Vousden, 2010)* are also described to promote glucose metabolism.

3. Proliferative advantages

Energetically speaking, aerobic glycolysis appears as a wasteful version of metabolism: incomplete oxidation of glucose to lactate yields only 5 % of the energy available from glucose. However, the persistence of this particular phenotype in primary and metastatic cancers, even in conditions of normoxia, indicates that this apparently counterproductive behavior can confer a significant advantage to cancer cells. A constitutive upregulation of glycolysis indeed makes cancer cells insensitive to transient and permanent hypoxic conditions *(Granchi et al., 2014)*. Moreover, enhanced glucose uptake constitutes a rapid way to produce ATP for proliferating cells *(Pfeiffer et al., 2001)*. Most importantly, glycolysis facilitates rapid cell division as it readily provides metabolic intermediates that can be shunted to anabolic pathways, in which they serve as precursors for macromolecules *(DeBerardinis et al., 2008; Lunt and Vander Heiden, 2011)* (figure 2).

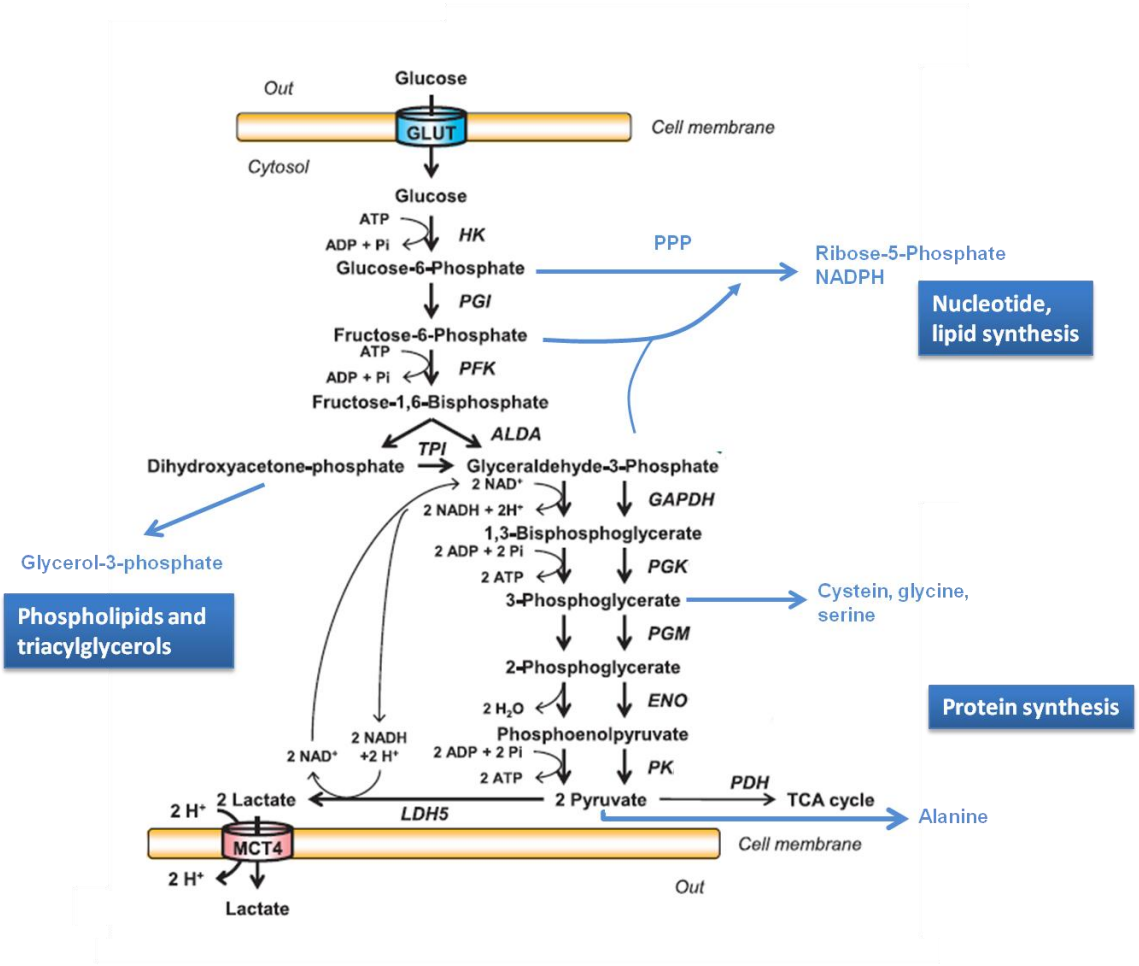

Figure 2: Glycolysis provides carbon intermediates that fuel the de novo production of lipids, nucleotides and amino acids, thereby promoting biomass expansion and cell division. GLUT: glucose transporter, HK: hexokinase, PGI: phosphoglucose isomerase, PFK: phosphofructokinase, ALDA: aldolase A, TPI: triosephosphate isomerase, GAPDH: glyceraldehyde-3-phosphate dehydrogenase, PGK: 3-phosphoglycerate kinase, PGM: phosphoglucomutase, ENO: enolase, PK: pyruvate kinase, LDH5: lactate dehydrogenase 5, MCT4: monocarboxylate transporter 4, TCA: tricarboxylic acid, PPP: pentose phosphate pathway. Adapted from *(Payen et al., 2015)*.

Glycolytic intermediates can fuel several biosynthetic pathways to generate de novo nucleotides, lipids and amino acids that are essential for the duplication of the cellular biomass during cell division. After its cellular uptake through glucose transporters (GLUTs), glucose is phosphorylated by HK, which produces glucose-6-phosphate (G-6-P). G-6-P can either continue to proceed into glycolysis, or can be irreversibly shunted to the oxidative branch of the pentose phosphate pathway (PPP) by G-6-P dehydrogenase (G6PD)

(Hamanaka and Chandel, 2012) (figure 3). The oxidative branch of the PPP is a major source of reduced nicotinamide adenine dinucleotide phosphate (NADPH), which protects against oxidative stress by electron transfer to thioredoxin/peroxiredoxin and glutathione systems, and which also serves as a cofactor necessary for macromolecules biosynthesis *(Jiang et al., 2014)*. Lipid synthesis requires a great amount of NADPH, which is readily evident when considering the 14 NADPH molecules needed for fatty acyl-CoA generation. As each phospholipid is composed of two molecules of fatty acyl-CoA, one can easily figure out that cell membrane synthesis require considerable high amount of NADPH *(DeBerardinis et al., 2008)*. The reversible non-oxidative branch of the PPP produces the nucleotide precursor ribose-5-phosphate (R-5-P), using either the product of the oxidative branch (ribulose-5-phosphate) or glycolytic intermediates *(Jiang et al., 2014)* (figure 3).

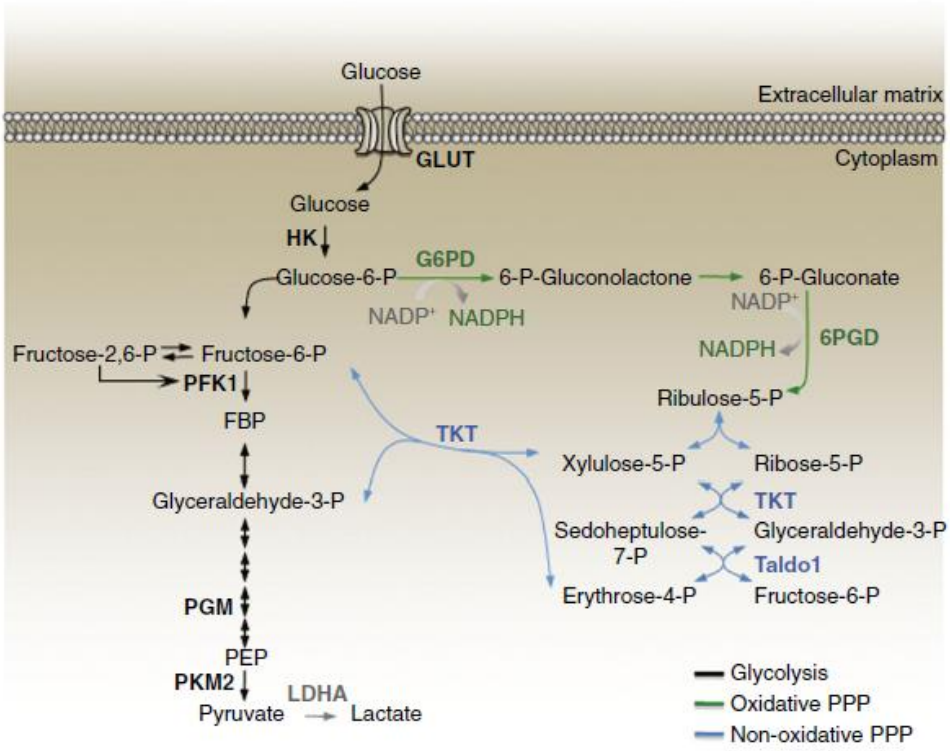

Figure 3: Using glucose-6-phosphate (glucose-6-P) as a substrate, the oxidative branch of the pentose phosphate pathway (PPP) produces NADPH that can be used in biosynthetic reactions for lipids and for antioxidant defense. The reversible non-oxidative branch of the PPP produces ribose-5-phosphate as well as glycolytic intermediates. G6PD: glucose-6-phosphate dehydrogenase, 6PGD: 6-phosphogluconate dehydrogenase, TKT: transketolase, Taldo1: transaldolase 1, HK: hexokinase, GLUT: glucose transporter, PFK1: phosphofructokinase-1, PGM: phosphoglyceratemutase, PKM2: pyruvate kinase M2, LDHA: lactate dehydrogenase A, FBP: fructose-1,6-bisphosphate, PEP: phosphoenolpyruvate. Jiang *et al.,* 2014.

Coming back to glycolysis, dihydroxyacetone-phosphate, a metabolic intermediate coming from the cleavage of fructose-1,6-bisphosphate, is the precursor of glycerol-3-phosphate, which is crucial for the biosynthesis of the phospholipids and triacylglycerols that serve as major structural lipids in cell membranes *(Lunt and Vander Heiden, 2011)*. Glyceraldehyde-3-phosphate and fructose-6-phosphate can combine to generate R-5-P, thereby contributing to nucleotide synthesis. Finally, protein synthesis can also be supported

by a high rate of glycolysis as the carbons of four amino acids are derived from two glycolytic intermediates: 3-phosphoglycerate, which provides carbons for cysteine, glycine and serine, and pyruvate, which provides carbons for alanine *(DeBerardinis et al., 2008; Hamanaka and Chandel, 2012)*.

In proliferating cells, carbons from pyruvate that enter the mitochondrial TCA cycle can also be diverted to biosynthetic pathways through the process of cataplerosis, therefore further participating to macromolecules synthesis. The role of mitochondria in redirecting energy-containing intermediates from ATP production towards biosynthesis is discussed in section III.2.

4. Consequences on the tumor microenvironment

Highly glycolytic cancer cells produce large amounts of lactate and protons that must be exported to avoid an intracellular acidification that would otherwise lead to cell death *(Dhup et al., 2012)*. Moreover, intracellular pH (pHi) must be kept in a narrow range, otherwise cell cycle progression and biosynthetic processes could be compromised *(Madshus, 1988)*. This can be achieved by monocarboxylate transporters (MCTs) that couple the export of each molecule of lactate with a proton *(Dimmer et al., 2000)*. Other important systems that catalyze proton exports include carbonic anhydrases (CAs), membrane-bound vacuolar ATPase (V-ATPase) and the sodium proton exchanger (NHE1) *(Dhup et al., 2012)*. CO_2 generated from various sources also contributes to tumor acidification *(Dhup et al., 2012)*. Indeed, through the action of CAIX, secreted CO_2 can be hydrated into bicarbonate (HCO_3^-) and H^+ *(Parks et al., 2011)*.

In cancer cells, all these systems result in a transmembrane gradient of protons opposite to that in normal cells; with a neutral or slightly alkaline intracellular pH ($pH_i \geq 7.4$) and an acidic extracellular pH (pH_e = 6.5-6.9) *(Gillies et al., 1994)*. As reviewed in *(Gatenby et al., 2006; Dhup et al., 2012; Estrella et al., 2013)* acidification has important role in tumor progression as it promotes invasiveness by reducing the proliferation and viability of

surrounding stromal cells, induces the breakdown of the extracellular matrix, promotes angiogenesis, and decreases immune responses to tumor antigens. Therefore, overexpression of the pH-regulating systems mentioned above is usually associated with poor prognosis *(Sennoune et al., 2004; Hussain et al., 2007; Chiang et al., 2008)*.

II. Targeting aerobic glycolysis

With the deepened understanding that enhanced glycolysis is a common feature of cancer cells and a key contributor to tumor growth and invasion, different approaches to tackle this Achilles' heel have been explored. Many compounds have been developed to selectively inhibit aerobic glycolysis in several tumor models *(Granchi et al., 2014)*. A recent update of drugs currently in clinical trials is available in Table 1.

The major concern associated with targeting cancer cell metabolism lies on the fact that all cells use the same metabolic network; and the disruption of any of these metabolic processes has the potential to affect both cancer and normal cells. Moreover, the majority of metabolic enzymes are not mutated *(Elf and Chen, 2014)*. Combination of drugs is therefore increasingly considered as an important avenue to be explored in order to reduce drug doses and spare normal cell. Moreover, dysregulated metabolism directly affects chemosensitivity via multiple pathways *(Zhao et al., 2013)*. The most significant advances and remaining challenges concerning (1) reduction of glucose entry into the cell, (2) inhibition of glycolytic enzymes or (3) redirection of pyruvate from lactate production are discussed below.

Target	Compound	Type of Cancer	Trial Status	Results	Obs	Present
GLUT1	Silybin					No Trial as monotherapy ongoing
		Prostate carcinoma	Phase I/II trial Completed	High toxicity Low pene-trance	--------	
		Hepatocellular carcinoma	Phase I/II trial Completed	--------	--------	
		Lung carcinoma	Phase II trial recruiting	--------	In combination with erlotinib	Ongoing
HKII	2-DG					No trial ongoing
		Breast, Lung, Pancreatic, Head and Neck and Gastric Cancer	Phase I trial completed	Clinically tolerable dose defined	In combination with docetaxel	
		Prostate cancer	Phase I/II trial suspended	Slow accrual	--------	
		Intracraneal Neoplasms Neoplasm metastasis	Phase I trial withdrawn	Pharmaceutical company ceased drug manufacturing	--------	
	Lonidamine					No trial ongoing
		Prostate carcinoma	Phase II/III suspended	Toxicity	--------	
PKM2						No trial ongoing
	TLN232	Refractory Metastatic renal cancer	Phase II trial completed	Drug safe and well tolerated	--------	
		Metastatic melanoma	Phase II trial suspended	--------	Dispute with licensor	
LDH	AT101					Trial ongoing in combination
		Chemotherapy-sensitive relapsed Small cell lung carcinomas	Phase I/II trial Completed	No efficacy	--------	
MCT1	AZD3965					Trial ongoing
		Prostate, Gastric cancer and Lymphomas	Phase I/II trial recruiting	--------	--------	
PDK	DCA					Multiple trials in combination
		Glioblastoma	Phase I trial completed	safe, well-tolerated and feasible	--------	
			Phase I trial completed	Good results	--------	
		Metastatic breast and Non-small cell lung cancers	Phase II/III suspended	--------	Safety concerns	
		Head and Neck cancers	Phase I recruiting			

Table 1: Update of clinical studies targeting glucose metabolism for cancer therapy.

From Granja *et al., 2015*.

1. Reduction of glucose entry into the cell

Due to the fundamental role of glucose uptake in the glycolytic pathway, targeting glucose transporters (GLUTS) is an attractive approach to inhibit tumor growth. Different GLUT isoforms (GLUT1, GLUT3 and GLUT4) have been found to be overexpressed in a wide variety of cancers *(Szablewski, 2013)* and their level of expression often correlates to bad prognosis *(Adekola et al., 2012)*. Silybin/silibinin is a natural compound that was recently shown to be a GLUT inhibitor *(Zhan et al., 2011)*. Pre-clinical *in vitro* and *in vivo* studies showing encouraging results *(Deep and Agarwal, 2010)* led to phase I and II clinical trials in patients with prostate cancer (NCT00487721) and advanced hepatocellular carcinoma (NCT01129570). No relevant anticancer effects were obtained because of a poor drug penetration in cancer tissues, which was accompanied by acute toxicity. However, as it was recently shown that silybin can reverse drug resistance and increase chemotherapeutic drug efficacy *(Cufi et al., 2013; Hussain and Marouf, 2013; Sadava and Kane, 2013)*, a phase II clinical trial was recently launched that will assess the efficacy of combined erlotinib with silybin (NCT02146118). Other natural (phloretin, (+)-cryptocaryone, methylxanthin) or synthetic (WZB117, STF-31, oxime derivatives) compounds inhibiting glucose transporters have shown promising pre-clinical results too, but the question of selectivity remains a major concern *(Granchi et al., 2014; Granja et al., 2015)*. The potential toxicity associated with glucose deprivation in healthy tissues that primarily depend on glucose metabolism (such as the brain) is indeed a major challenge for their use *in vivo*. The crystal structure of human GLUT1 was very recently obtained *(Deng et al., 2014)*, which will most probably help to develop new selective inhibitors.

2. Inhibition of glycolytic enzymes

Owing to the fact that they catalyze reactions essentially irreversible, efforts have mainly focused on the specific inhibition of HK, PFK and PK, the three rate-limiting enzymes of glycolysis. Although less characterized or still under development, compounds targeting

other enzymes in glycolysis, such as phosphoglycerate mutase (PGM) and enolase (ENO), are also under investigation *(Granchi et al., 2014)*.

2.1 Hexokinase

HK is the first rate-limiting enzyme of the glycolytic pathway that phosphorylates glucose to yield G-6-P, trapping glucose inside the cell (figure 2). The glucose analogue 2-deoxy-D-glucose (2-DG) is the most widely used and advanced clinical agent inhibiting cancer metabolism *(El Mjiyad et al., 2011)*. 2-DG is phosphorylated by HK to generate 2-DG-6-P but it cannot be further metabolized, therefore inhibiting HK activity non-competitively. Although 2-DG is mainly described as a HK inhibitor, 2-DG-6-P also acts as a competitive inhibitor of enzymatic reactions that use G-6-P as a substrate, therefore inhibiting both glycolysis and the PPP. 2-DG may also compete with glucose for GLUT transporters, therefore decreasing glucose uptake by the cell *(Granja et al., 2015)*. The anticancer effects of 2-DG include inhibition of proliferation and colony formation *in vitro*, as well as *in vivo* tumor growth inhibition *(Laszlo et al., 1960; Kern and Norton, 1987; Bell et al., 1998)*. Moreover, induction of apoptosis, autophagy, proteolytic events and endoplasmic reticulum stress were also reported *(Kuntz et al., 2014)*. Upon determining the clinically tolerable dose, a completed phase I clinical trial reported significant adverse effects associated to 2-DG delivery (63-88 mg/kg), such as hyperglycemia, gastrointestinal bleeding and cardiac abnormalities (NCT00096707). It was realized that to achieve therapeutic effects, high doses of 2-DG would be required. In view of the risk of toxicity, other trials (NCT00247403 and NCT00633087) that aimed to treat cancer with 2-DG were discontinued. *In vitro* studies using a combination of 2-DG with standard therapeutic agents such as cisplatin and docetaxel showed that 2-DG may potentiate the effect of standard therapy *(Maschek et al., 2004; Ciavardelli et al., 2014)*. 2-DG also sensitized gliomas and other cell lines to ionizing radiations without inducing relevant side effects *(Khaitan et al., 2006; Sharma et al., 2012)*.

The HK family comprises 4 isoforms, with isoform II (HKII) predominantly overexpressed in malignant lesions *(Shinohara et al., 1994; Pedersen et al., 2002)*. HKII has the particularity to be located at the outer membrane of mitochondria, where it interacts with the voltage dependent anion channel (VDAC). Upregulation of this mitochondria-bound isoform offers a preferential access of HK to newly synthesized ATP for glucose phosphorylation, and contributes to inhibition of apoptosis through blocking of cytochrome C release *via* VDAC *(Mathupala et al., 2009)*. Importantly, most normal mammalian tissues express very little HKII which suggests that only limited side-effects are expected from compounds selectively targeting HKII *(Vander Heiden, 2011)*. Lonidamine is a synthetic compound described as a specific inhibitor of mitochondrial-bound HK *(Floridi et al., 1981)* that has shown promising anticancer effects in various tumor types (reviewed in *(Di Cosimo et al., 2003)*). Until recently, lonidamine was in phase II/III clinical studies for the treatment of patients with prostate cancer (NCT0435448 and NCT00237536); however, the trials have been suspended due to liver toxicity. An inhibitory activity on HKII was also demonstrated for anticancer agents with multiple mechanisms of action, such as 3-bromo-pyruvate *(Mathupala et al., 2009)* and metformin *(Salani et al., 2013)*. Besides this, a pharmaceutical company (vTvtherapeutics) and scientists of the Okinawa Institute of Science And Technology *(Cui and Tanaka, 2013)* are currently developing and studying the effects of novel specific HKII inhibitors, which could lead to interesting results in the near future.

2.2 Phosphofructokinase

PFK-1 catalyzes one of the most critical steps of glycolysis, the conversion of fructose-6-phosphate and ATP to fructose-1,6-bisphosphate and ADP (figure 2). The activity of PFK-1 is highly regulated, with fructose-2,6-bisphosphate acting as a powerful allosteric activator. As the main provider of fructose-2,6-bisphosphate, PFKFB3 (a member of the PFK-2 family) is an important contributor to the high glycolytic activity of cancer cells *(Ros and Schulze, 2013)*. Its activity can be reduced with the selective inhibitor 3PO (3-(3-

pyridinyl)-1-(4-pyridinyl)-2-propen-1-one) *(Clem et al., 2008)*. 3PO was reported to decrease the concentration of fructose-2,6-bisphosphate, leading to decreased glucose uptake and to cancer growth suppression *in vitro* and *in vivo (Clem et al., 2008).* Very recently, further optimization of this small molecule led to the synthesis of PFK158, a more potent inhibitor of PFKFB3 for testing in the clinics. The phase I clinical trial has started in March 2014 and is presently recruiting participants (NCT02044861).

2.3 Pyruvate kinase

PK catalyzes the final rate-limiting step of glycolysis, which consists in the transfer of a phosphate group from phosphoenolpyruvate (PEP) to ADP, to yield pyruvate and ATP (figure 2). The M1 isoform is expressed in normal proliferating cells, whereas the M2 isoform is expressed during embryonic development and in many cancers *(Christofk et al., 2008)*. PKM2 is the only PK to be allosterically regulated between a less active dimer and an active tetramer *(Wong et al., 2013)*. These different forms of PKM2 regulate glucose metabolism through either the TCA cycle or glycolysis. The dynamic equilibrium between dimer and tetramer PKM2 allows proliferating cells to regulate their needs for anabolic and catabolic metabolism. In 2007, Thallion Pharmaceuticals launched the first clinical trial (phase II) with the PKM2 inhibitor TLN-232 (NCT00422786) in patients with refractory metastatic renal cell carcinoma. The drug was generally safe and well tolerated, with 2 out of 3 patients that completed the study with a stable disease *(Granja et al., 2015)*. A second phase II clinical trial for the treatment of metastatic melanoma patients was suspended in 2010 for legal reasons (NCT00735332). Approaches that enhance PKM2 activity to promote OXPHOS have also been considered. In preclinical studies, PKM2 activators showed inhibitory effects on cancer cells proliferation and tumorigenesis *(Anastasiou et al., 2012)*.

3. Redirecting the fate of pyruvate from lactate production toward mitochondrial oxidation

3.1 Lactate dehydrogenase

LDHs belong to a family of tetrameric enzymes that are formed by two different subunits, M and H, assembled to form active tetramers. Five distinct enzymes, LDH1 to LDH5, therefore exist *(Porporato et al., 2011)*. LDH1 is ubiquitously expressed in normal tissues and preferentially catalyzes the reduction of lactate to pyruvate. Conversely, LDH5 is highly expressed in many human tumors and preferentially catalyzes the opposite reaction; oxidizing pyruvate to lactate (figure 2) *(Koukourakis et al., 2009)*. LDH5 also allows to maintain a high rate glycolytic flux by generating NAD^+, a cofactor required for the reaction catalyzed by the glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The antimalarial natural compound gossypol (AT101) competes with NADH and inhibits LDH5 with a low Ki (1.9 µM) *(Granja et al., 2015)*. Unfortunately, clinical studies using AT101 in monotherapy (NCT00773955) or in combination with chemotherapy (NCT00397293) revealed disappointing results or were interrupted. New studies with AT101 in combination with other drugs are starting to recruit patients (NCT01003769 and NCT01977209). FX11, a gossypol-derived compound has shown interesting anti-proliferative properties *in vitro* and *in vivo (Le et al., 2010)*. No clinical evaluation of FX11 has been initiated yet, but it would maybe provide new opportunities to target LDH5, although significant toxicity and off-target effects are expected *(Granchi et al., 2014)*.

3.2 Pyruvate dehydrogenase kinase

PDK is a mitochondrial kinase that negatively regulates the activity of pyruvate dehydrogenase (PDH), the enzymatic complex responsible for the irreversible conversion of pyruvate to acetyl-CoA *(Holness and Sugden, 2003)* (figure 4). By phosphorylating PDH, PDK effectively blocks PDH activity, thus preventing pyruvate entry into the TCA cycle and forcing the cells to switch to glycolysis for energy production.

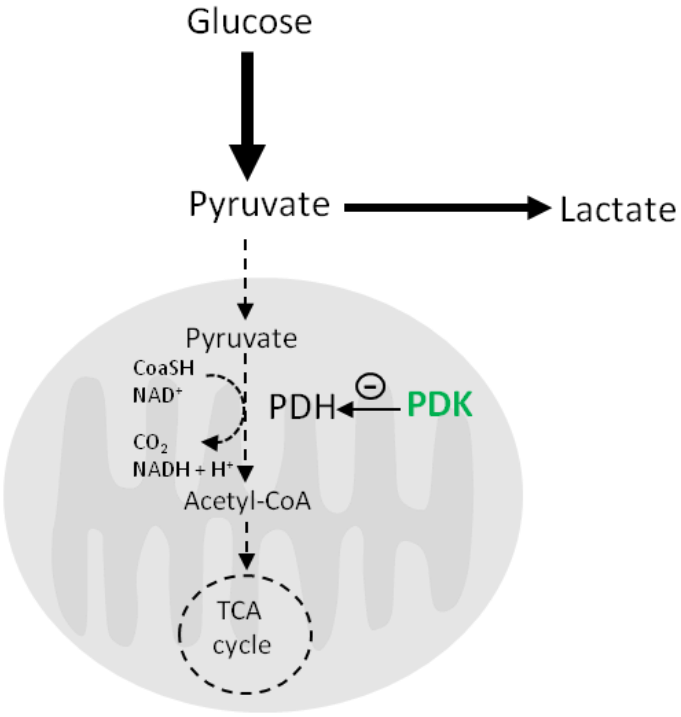

Figure 4:
The intramitochondrial pyruvate dehydrogenase kinase (PDK) inhibits pyruvate dehydrogenase (PDH) activity through phosphorylation, thereby inhibiting the metabolization of pyruvate in mitochondria.

PDK is reversibly regulated by the products (acetyl-CoA and NADH) and substrate (pyruvate) of the PDH reaction *(Bowker-Kinley et al., 1998)*. There are four human isomeric forms of PDK (PDK1-4), with tissue-specific expression and singular activities *(Patel et al., 2014)*. PDK1 *(Kim et al., 2006; Papandreou et al., 2006; McFate et al., 2008; Kaplon et al., 2013)* and PDK3 *(Lu et al., 2008)* are known to be target gene products of HIF-1 and have been found expressed in cancer cells. PDK2 is also expressed in many tumors *(Bonnet et al., 2007; Michelakis et al., 2010)* and has been found to be repressed by the tumor suppressor gene p53 *(Contractor and Harris, 2012)*. PDK4 can be activated by the oncogenic Rb/E2F1 pathway, suggesting a role of PDK4 in tumor biology *(Hsieh et al., 2008)*.

3.2.1 Targeting PDK with dichloroacetate

Because of their strategic location, PDKs have already been considered as important therapeutic targets. Dichloroacetate (DCA) was identified as a PDH activator 40 years ago *(Whitehouse and Randle, 1973)*. It is now known that this pyruvate mimetic acts

by inhibiting the action of the PDKs. The crystal structure of PDK2 bound to DCA has been obtained, and it appears that DCA occupies the pyruvate-binding site in the N-terminal regulatory domain *(Knoechel et al., 2006)*. The four isozymes vary considerably in their sensitivity to inhibition by DCA. PDK2 is the most sensitive, PDK3 the most resistant, whereas the sensitivities of PDK1 and PDK4 are intermediate *(Bowker-Kinley et al., 1998)*.

3.2.2 Dichloroacetate as an anticancer treatment

DCA has already been used in humans as a treatment for congenital lactic acidosis *(Stacpoole et al., 1992)*. In these patients, pyruvate cannot be oxidized in the mitochondria because of a lack or a decreased expression of mitochondrial enzymes, and pyruvate is metabolized to lactate by LDH. In 2007, Bonnet and coworkers *(Bonnet et al., 2007)* first reported the anticancer properties of DCA. They showed that DCA changes the metabolism of cancer cells from cytoplasm-based glycolysis to mitochondria-based oxidation. This was associated with an increased production of reactive oxygen species (ROS), decreased mitochondrial membrane potential, decreased efflux of proapoptotic mediators from mitochondria (cytochrome C and apoptosis-inducing factor [AIF]), and induction of apoptosis. *In vivo*, daily oral administration of DCA reduced tumor growth. The authors also showed that cancer cells are more sensitive to DCA treatment because they exhibit hyperpolarized mitochondria, which is thought to confer resistance to apoptosis.

Based on the results of this initial study by Bonnet *et al*; DCA has quickly been evaluated as a potent anticancer drug in the clinics. Indeed, its structure (a very small molecule able to cross the blood-brain barrier), its low price (it is a generic drug) and the fact that DCA has already been used in humans provided a strong rationale for rapid clinical evaluation *(Michelakis et al., 2008)*. In 2010, outcomes of the first clinical trial in five patients with glioblastoma were available (NCT00540176). DCA treatment was associated with a prolonged stabilization or tumor regression and, in general, displayed an overall good safety profile. Dose-limiting toxicity was a peripheral neuropathy that was reversible upon DCA

dose reduction *(Michelakis et al., 2010)*. Another phase I trial (NCT01111097) in patients with glioblastoma or recurrent brain tumor (completed in 2014) showed that oral DCA administration is safe, well-tolerated and feasible. In this trial, dosing was based on haplotype variation in glutathione transferase zeta 1 (GSTZ1), which participates in DCA catabolism. Consequently, no dose-limiting toxicity occurred. No patient withdrew because of lack of tolerance to DCA. Importantly, DCA therapy was associated with clinical and radiographic evidence of disease stabilization in eight out of fifteen patients *(Dunbar et al., 2014)*. Conversely, a phase II trial conducted in patients with refractory metastatic breast and non-small cell lung cancers (NCT01029925) was prematurely terminated based on higher than expected risk and safety concerns. To date, two other clinical studies using DCA in monotherapy are ongoing and could provide more information about the clinical use of DCA in cancer therapy (NCT00566410 and NCT01163487). One trial using oral DCA in combination with cisplatin and radiation treatment in patients with head and neck carcinoma is ongoing (NCT01386632).

3.2.3 Other mechanisms attributed to dichloroacetate

Since the initial work by Bonnet *et al.,* DCA has been widely studied alone or in combination with other treatments, as reviewed in *(Kankotia and Stacpoole, 2014)*. From these *in vitro* and *in vivo* studies, it was generally concluded that induction of ROS-mediated apoptosis is the main mechanism accounting for the tumoricidal action of DCA. Nevertheless, some groups recently reported distinct mechanisms, and this hypothesis has been questioned. Using a panel of sixteen cancer and normal cell lines, a systematic study of Stockwin and coworkers *(Stockwin et al., 2010)* highlighted that DCA is relatively inactive, inducing apoptosis only at high concentrations (≥ 25 mM, 48 h). At lower concentrations, DCA depolarizes mitochondria, increases ROS generation and inhibits cell growth without apoptosis induction. Interestingly, increased ROS levels were not implicated in the anti-proliferative effects of DCA. It was rather suggested that, at clinically relevant doses, DCA is

a metabolic modulator that negatively impacts cell growth without apoptosis induction. Another study, that used hypoxic HeLa cervical and PANC-1 pancreatic cancer cells to evaluate the *in vitro* effects of DCA reported that 94 % of the cells were viable at doses of 12.5 mM (24 h treatment) *(Anderson et al., 2009)*. In HT29 and LoVo cell lines isolated from colorectal adenocarcinomas, there were increases of 2.8 % and 21 % in total apoptotic cells following 50 mM of DCA treatment for 48 h, respectively *(Madhok et al., 2010)*. Similarly, in two other recent studies, an apoptotic response was triggered at concentrations of DCA that were too high to be clinically relevant *(Abildgaard et al., 2014; Delaney et al., 2015)*. Taken together, these reports show that DCA can have different actions that may likely depend on drug concentration and cell type.

III. Targeting mitochondria

Oxidative metabolism and TCA cycle functions in cancer cells have been the subject of intense debate. This is in part due to Warburg's hypothesis claiming that the origin of cancer was a permanent, irreversible impairment of OXPHOS *(Warburg, 1956)*. However, metabolic flux experiments tracking carbon-labeled nutrients have shown that the TCA cycle remains intact in cancer cells exhibiting a pronounced Warburg phenotype *(DeBerardinis et al., 2007)*. In addition, a significant population of cancer cells may predominantly rely on OXPHOS for ATP production *(Zu and Guppy, 2004; Moreno-Sanchez et al., 2014)*. Therefore, increasing evidence indicates that mitochondrial activity is not fundamentally impaired, but rather actively participates in the transformation process by maintaining the biosynthetic capacity of cancer cells. As discussed below, several strategies consisting in inhibiting mitochondria alone or in combination with cytotoxic modalities such as radiotherapy have already been evidenced to constitute appealing approaches for cancer therapy.

1. Electron transport chain and oxidative phosphorylation

The initial statement made by Warburg on the origin of cancer was immediately criticized with the demonstration by Sidney Weinhouse that the mitochondrial function in cancer tissues was most often normal and that information supporting Warburg's hypothesis was affected by misinterpretation *(Weinhouse, 1956)*. In fact, analysis of the degree of inhibition of glycolysis by metabolic intermediates including ATP derived from OXPHOS (the "Pasteur Effect") showed that aerobiosis can reduce glycolysis in most cancer cells, indicating a normal oxidative capacity in tumors *(Weinhouse, 1976)*. More recent experimental studies have also provided evidence that mitochondrial activity is not fundamentally impaired in many cancer cells:

- Diverting pyruvate into the mitochondria through inhibition of LDH *(Fantin et al., 2006)* or activation of PDH by targeting its inhibitor PDK with DCA *(Bonnet et al., 2007)* can significantly induce respiration in cancer cells.
- Under glucose starvation, most cancer cells can survive when making ATP exclusively by OXPHOS *(Rossignol et al., 2004)*
- Despite enhanced rates of glycolysis, Zu and Guppy *(Zu and Guppy, 2004)* showed that OXPHOS was the main ATP supplier in an extensive number of cancer cell lines.
- A point that is often overlooked is that re-oxidation by the ETC of NADH formed by TCA cycle enzymes or by glycolysis in the cytosol is required for the continuous supply of citrate, the only source for the synthesis of cytosolic lipogenic precursor acetyl-CoA (see III.2 for more details) *(Moreno-Sanchez et al., 2014)*.

Overall, there are still some examples of tumor cell lines that exhibit decreased mitochondrial functions caused by mutations in either mitochondrial-encoded or in nuclear-encoded mitochondrial proteins *(Carew and Huang, 2002; Chatterjee et al., 2006; Frezza et al., 2011)*. As examples, the TCA cycle enzymes isocitrate dehydrogenase (IDH), succinate dehydrogenase (SDH) and fumarate hydratase (FH) have been found deleted or mutated in various forms of cancer. Mutations affecting respiratory chain complexes have also been reported. But growing evidence suggests that most cancer cells are capable of performing OXPHOS. As was hypothesized by Frezza and Gottlieb *(Frezza and Gottlieb, 2009)*, decreased OXPHOS could in many cases rather be a consequence of accelerated glycolysis and an increased use of pyruvate for lactate production driven by genetic and/or environmental alterations. A suggested hypothesis is that sustained respiration inhibition by glycolysis is caused by glycolysis competing with oxidative OXPHOS for inorganic phosphate (Pi) and ADP.

Preclinical and clinical evaluation of several compounds targeting mitochondrial respiration has shown encouraging results. Resveratrol is a natural compound found in grapes that binds to ATP synthase and inhibits ATP synthesis. Research using cancer cells has shown promising and positive effects of resveratrol implicating different mechanisms. However, evidence from rodents and humans is inconsistent *(Patel et al., 2010; Carter et al., 2014)*. Retrospective clinical and laboratory evidence further strongly suggests that the antidiabetic drug metformin acts as an anticancer agent *(Evans et al., 2005; Dowling et al., 2012)*. The tumoricidal effects of metformin are not entirely clear, but studies demonstrated that metformin when used *in vitro* inhibits mitochondrial complex I *(El-Mir et al., 2000; Owen et al., 2000)* and *in vivo* models have shown that the antitumor effects of metformin are dependent on its ability to inhibit this complex *(Fendt et al., 2013; Wheaton et al., 2014)*. Other reports using mitochondrial inhibitors that selectively target ATP transport or OXPHOS processes can be found in these reviews *(Ramsay et al., 2011; Wen et al., 2013; Weinberg and Chandel, 2015)*. For the moment, only few molecules progressed beyond preclinical investigations.

2. TCA cycle

In non-proliferating cells, a main role of the TCA cycle is to fuel ATP production. In proliferating cells such as cancer cells in growing tumor areas, however, the TCA cycle serves as an important source of biosynthetic precursors in addition to providing ATP *(DeBerardinis et al., 2008; Frezza and Gottlieb, 2009)* (figure 5):

- TCA cycle-derived citrate is the only source for the synthesis of cytosolic acetyl-CoA required for lipid synthesis.
- Oxaloacetate (OAA) and α-ketoglutarate supply intracellular pools of non-essential amino acids (aspartate, asparagine, glutamate, proline, arginine) used for the production of proteins and nucleotides

- Malate, once exported from mitochondria, serves to produce NADPH and pyruvate, a precursor of alanine.

In order to sustain TCA cycle functions and face cataplerosis (depletion of TCA cycle intermediates supporting biosynthesis), cells must have a matching influx of intermediates that replenish the cycle (anaplerosis). An important source of anaplerosis is the metabolism of glutamine, the most abundant amino acid in mammals *(DeBerardinis et al., 2007)*. In the cytosol, glutamine donates nitrogen to purines and pyrimidines, resulting in the formation of glutamate. Glutamate produces non-essential amino acids and α-ketoglutarate, which can replenish the TCA cycle in carbon intermediates. In addition, cells can partially oxidize α-ketoglutarate derived from glutamine *(Reitzer et al., 1979)*, which increases the cytosolic production of NADPH and pyruvate, a precursor of alanine.

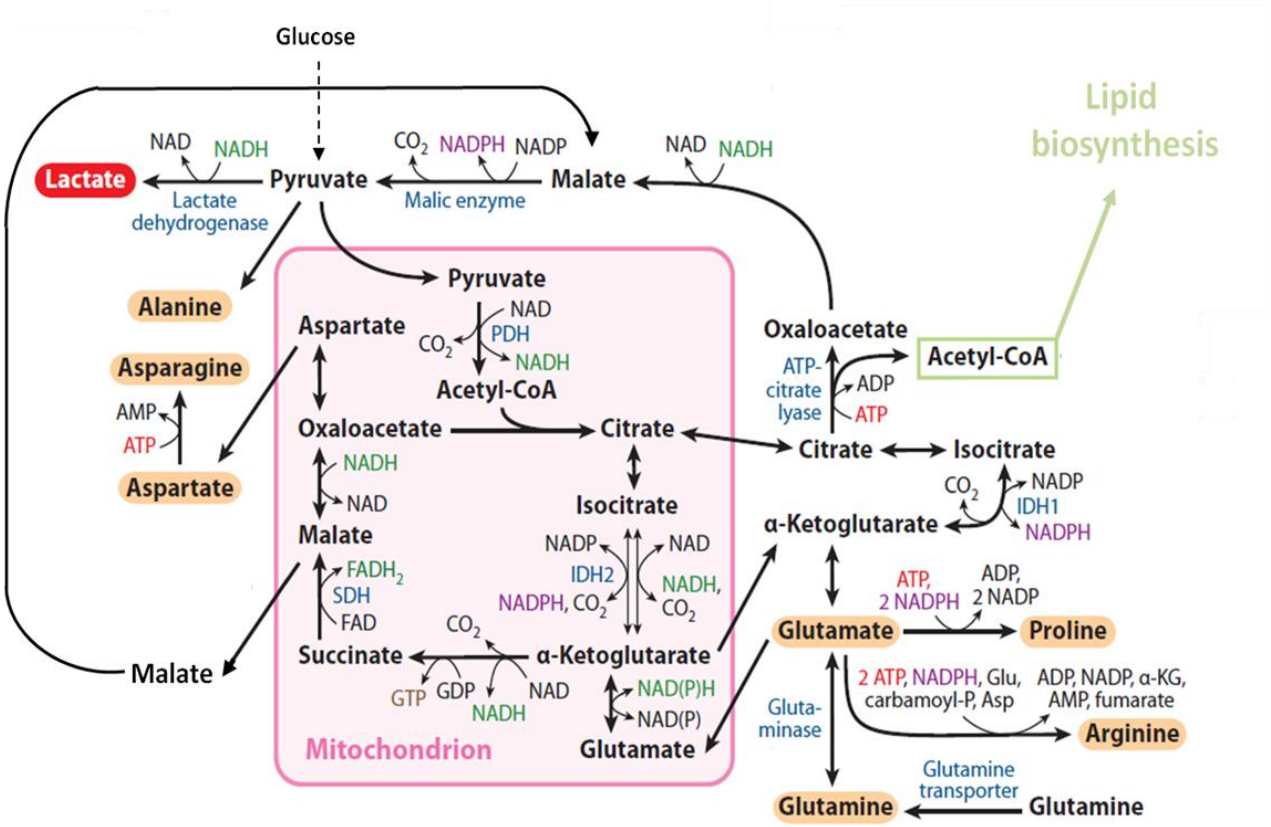

Figure 5: Proliferating cells such as cancer cells can use TCA cycle intermediates as precursors for biosynthesis. Glucose and glutamine are two important carbon sources that fuel the TCA cycle. They are converted into citrate that is exported to the cytosol to participate in the synthesis of fatty acids and cholesterol. Oxaloacetate and α-ketoglutarate are also used to generate non-essential amino acids. α-ketoglutarate derived from glutamine is ultimately sequentially converted to malate, pyruvate and lactate, generating additional NADPH (the malic enzyme reaction), NAD^+ and lactate (the lactate dehydrogenase reaction). Adapted from Vander Heiden and Lunt., 2011.

Although anaplerosis can be achieved by pyruvate and glutamine, increasing evidence suggests that glutamine may predominate over the use of pyruvate in many cancer cells *(Medina et al., 1988; Souba, 1993; DeBerardinis et al., 2007)*. On the other hand, using stable isotope labeling coupled with nuclear magnetic resonance analysis, a group showed that orthotopically implanted human glioblastoma cells preferentially use glucose – rather

than glutamine – to produce TCA cycle intermediates *(Marin-Valencia et al., 2012)*. Undoubtedly, metabolic constraints imposed by the *in vivo* environment profoundly influence the way cancer cells metabolize substrates. Nevertheless, targeting glutamine metabolism has already shown therapeutic potential in preclinical studies. For example, aminooxyacetate (AOA) is a global inhibitor of transaminases that are involved in amino acid metabolism. It was reported to deplete both aspartate and alanine, decrease proliferation of cancer cells, and suppress tumor growth in mice *(Thornburg et al., 2008; Wise et al., 2008; Korangath et al., 2015)*. Glutamine analogs have also been explored as possible therapies for cancer. The antitumor effect of these compounds is believed to be exerted by inhibiting glutamine-dependent biosynthesis. Development of acivicin, 6-diazo-5-oxo-L-norleucine, and azaserine, three of the most widely studied glutamine analogs, has however been halted because of lack of tumor specificity and significant side-effects such as neurotoxicity, gastrointestinal toxicity and myelosuppression *(Wise and Thompson, 2010; Hensley et al., 2013)*. A more selective approach could be obtained by targeting SLC1A5, the transporter implicated in glutamine uptake by cancer cells. It has been shown that γ-L-glutamyl-p-nitroanilide (GPNA) inhibits this transporter and limits lung cancer cell growth *(Hassanein et al., 2013)*.

3. Sensitization to radiotherapy

Radiation therapy (RT) plays an important role in the care of patients with cancer. For example, approximately 75% of patients with head and neck cancer do benefit from RT as part of their treatment *(Barton et al., 2014)*. However, RT efficacy is severely compromised by hypoxia, a common feature of solid tumors. In the following section, we will discuss the influence of hypoxia on the radioresponse of tumors. The radiosensitizing approach that consists in increasing tumor oxygenation by inducing a decrease in mitochondrial O_2 consumption will be emphasized.

3.1 Effect of tumor hypoxia on the radioresponse of tumors

Hypoxia results from an imbalance between O_2 supply and consumption within a tissue. In solid tumors, structural and functional vascular abnormalities lead to insufficient O_2 supply to cells with high metabolic activities. Accordingly, O_2 partial pressure (pO_2) values of 2.5 mmHg or less are characteristic of advanced solid tumors in a wide range of human cancers *(Tatum et al., 2006)*.

It has been recognized for approximately 60 years that hypoxia profoundly decreases RT efficacy and is associated with poor clinical outcome. O_2 tensions below 10 mmHg are estimated to significantly reduce radiosensitivity *(Thomlinson and Gray, 1955)*. It is related to the fact that O_2 generates superoxide and fixes DNA damage following radiation treatment *(Jordan and Sonveaux, 2012)* (figure 6). Therefore, strategies that increase the delivery and/or decrease the metabolic utilization of O_2 can potentially render tumors more sensitive to radiation.

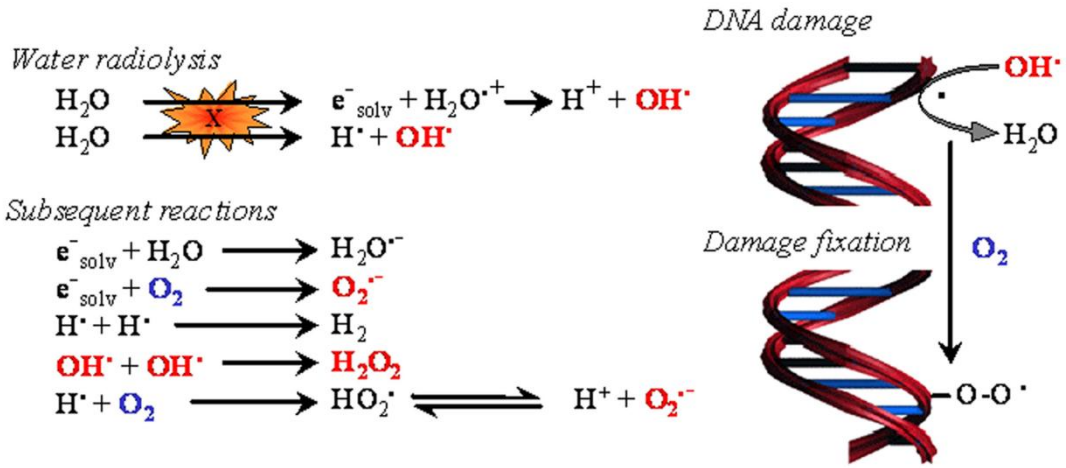

Figure 6: Oxygen potentiates RT efficacy. Oxygen is required for the generation of superoxide following water radiolysis. It is also important to stabilize ROS-generated DNA peroxides,i.e., stable DNA lesions that cannot be easily repaired. Jordan and Sonveaux, 2012.

Most attempts to reduce tumor hypoxia have focused on increasing the O_2 supply. However, these trials have met limited success. Indeed, combination of RT with approaches consisting in breathing O_2-enriched gases showed limited clinical impact *(Higgins et al., 2015)*. Nimorazole, which acts as an O_2 mimetic, showed decreased locoregional recurrence rates but failed to improve the survival of patients with head and neck cancer *(Overgaard et al., 1998)*; so that these findings have had no impact on general clinical practice except for Denmark. Studies on strategies that improve tumor oxygenation by achieving vasodilatation (the “provascular” approach) have provided encouraging data. However, it is important to note that because the proportion of mature vasoactive blood vessels is limited in solid tumors, vasodilators must be tumor-selective in order to increase tumor perfusion. Nonselective vasodilation would rather decrease tumor perfusion and oxygenation because blood would ooze out of the tumor to the normal tissues with lower vascular resistance to flow (“Steal Effect”) *(Danhier et al., 2013)*.

3.2 Reducing the oxygen demand to improve radiosensitivity

An alternative approach to increasing O_2 delivery to tumors to reduce tumor hypoxia is to decrease the O_2 demand by mitochondria, which account for most of cellular O_2 consumption. It has not yet attracted great interest in clinical trials but several arguments concur to indicate that targeting mitochondrial metabolism appears to be a good strategy to radiosensitize tumors:

- It is now recognized that most cancer cells have active OXPHOS, even if this activity is limited in highly glycolytic cells.
- Experimental evidence indicates that respiratory complex IV (cytochrome C oxidase) *Km* value for O_2 is in the range of 1 µM, thus ETC is able to function optimally at pO_2 values between 2.5 - 10 mmHg (equivalent to 3-13 µM O_2) *(Scandurra and Gnaiger, 2010)*. Consequently, mitochondrial inhibitors may allow sparing O_2 even in hypoxic tumor regions.

- It has been mathematically demonstrated *(Secomb et al., 1995)* and experimentally validated *(Diepart et al., 2012)* that targeting O_2 consumption is a very effective way to reduce tumor hypoxia.

Several pharmacological drugs that inhibit O_2 consumption have been characterized preclinically for their potential to increase tumor oxygenation and enhance the radiosensitivity of tumors (reviewed in *(Jordan and Sonveaux, 2012)*). A recent paper further showed that metformin can reduce the O_2 consumption rate and radiosensitize cancer cells by blocking complex I activity in the ETC. Metformin reduced tumor hypoxia in xenograft models without altering blood flow, and caused marked tumor regrowth delay following irradiation *(Zannella et al., 2013)*. Metformin combined with radiotherapy is likely to be well tolerated, since numerous diabetic patients with cancer have already received these treatments concurrently without any reported safety concerns *(Higgins et al., 2015)*.

3.3 Nitric oxide: a small molecule with multiple radiosensitizing mechanisms

Nitric oxide (NO) is an endogenous gas, the use of which represents a feasible and safe therapeutic option for radiotherapy. Treatments acting either by exogenous administration of NO donors or by the stimulation of endogenous production revealed that NO can radiosensitize tumors by at least 3 mechanisms:

- ***Vasodilation****:* Systemic administration of exogenous NO with isosorbide dinitrate, xanthinol nicotinate or *S*-nitrosocaptopril resulted in an improvement of oxygenation in experimental tumors due to an increase in tumor blood flow *(Jordan et al., 2000; Jordan et al., 2010; Segers et al., 2010)*. In the same line, electrical stimulation of endogenous NO synthesis also increased tumor blood flow and pO_2. This was attributed to the fact that NO acts as a major endothelium-derived relaxing factor (EDRF) *(Palmer et al., 1987)*. All treatments were efficient at radiosensitizing tumors.

- ***Inhibition of oxygen consumption****:* Some of the treatments cited above concomitantly decreased the rate of O_2 consumption by tumor cells. Indeed, NO also reversibly inhibits complex IV *(Brown and Cooper, 1994)*. Inhibition of tumor cell O_2 consumption has also been described in the context of insulin-induced NO-dependent enhanced radiosensitivity *(Jordan et al., 2002)*.
- ***RT damages stabilization****:* The mismatch between the amplitude of the tumor reoxygenation and the radiotherapeutic response *in vivo* suggested that NO also possesses intrinsic radiosensitizing properties *(Jordan et al., 2004)*. Hence, it has been proposed that NO acts as an intrinsic radiosensitizer because of its radical nature and its direct participation to the stabilization of DNA damage *(Mitchell et al., 1993; Griffin et al., 1996)*.

IV. Hydrogen sulfide

1. Endogenous production in mammals

Until recently, H_2S was seen almost exclusively as a toxic gas devoid of physiological significance. After carbon monoxide (CO) and nitric oxide (NO), H_2S is now considered the third member of the gaseous transmitter family in mammals *(Wang, 2002)* (figure 7).

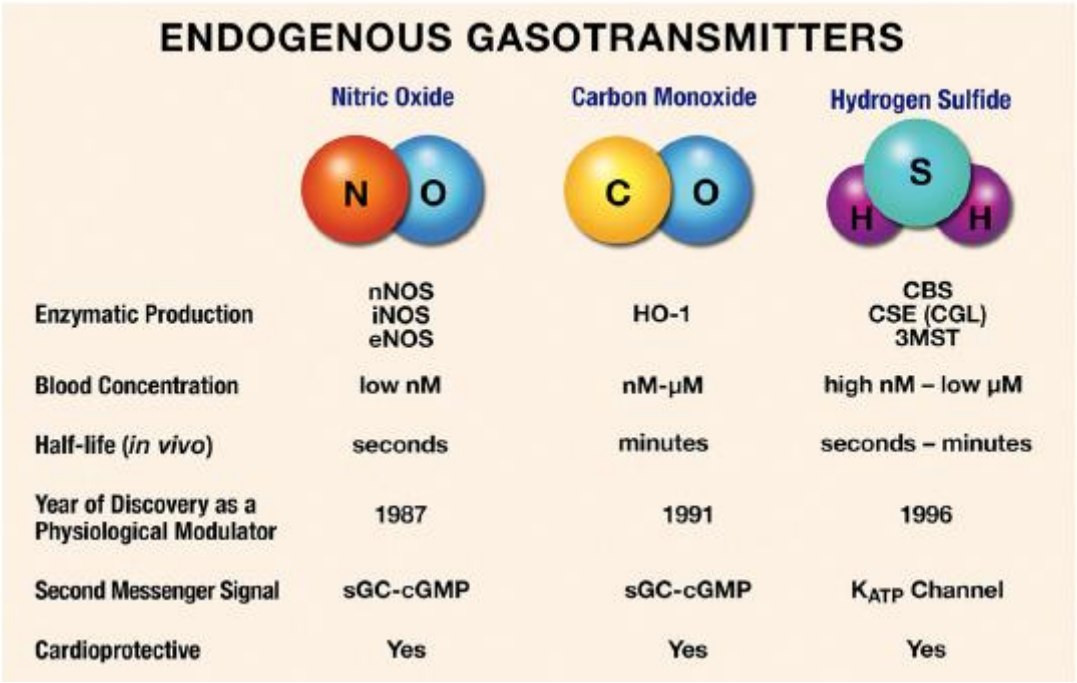

Figure 7: General characteristics of nitric oxide (NO), carbon monoxide (CO) and hydrogen sulfide (H_2S), the three recognized gasotransmitters in mammals. *(Polhemus and Lefer, 2014)*.

H_2S is a small gaseous molecule that is freely permeable to membranes. It is enzymatically generated in the body, and its endogenous metabolism is regulated. Among the enzymes involved in the endogenous production of H_2S, CBS (cystathionine-β-synthase) and CSE (cystathionine-γ-lyase) account for the major portion of H_2S. These enzymes use amino acids L-cysteine, L-homocysteine and L-cystathionine to produce H_2S with pyridoxal

5'-phosphate (vitamin B6) as a cofactor *(Wang, 2012)*. In addition, a minor endogenous source of H_2S is the non-enzymatic reduction of elemental sulfur to H_2S *(Wang, 2012)*. The physiological concentrations of H_2S range from high nM up to about 100 µM in the blood circulation of animals and humans *(Richardson et al., 2000; Hyspler et al., 2002)*.

2. Interactions with mitochondria

An important characteristic of H_2S is that, under certain circumstances, it may induce opposite biological responses. Mitochondria are important targets of H_2S and interactions are complex. Indeed, H_2S produces biphasic effects on cell respiration; inhibiting it at 10 µM and above but stimulating it at lower concentrations. The following section will describe how H_2S can act as an inhibitor or a stimulator of the ETC.

2.1. Inhibitory effects of H_2S on mitochondrial respiration

Similar to NO and CO, the primary molecular target of H_2S is the mitochondrial complex IV *(Petersen, 1977)*. Using a synthetic model of cytochrome C oxidase (Cox), Collman and colleagues *(Collman et al., 2009)* showed that at higher concentration (approximately 10 µM and above), H_2S binds to the iron center (Fe^{2+}) in the reduced active site and acts as an inhibitor of the enzyme as it competes with O_2. The inhibitory effect of H_2S on mitochondrial respiration has been well documented in various cell lines *(Khan et al., 1991; Thompson et al., 2003; Buckler, 2012)*. This inhibition is reversible because O_2 can easily replace bound H_2S; with Cox activity returning to its original levels within 10 - 30 min *in vitro (Di Meo et al., 2011)*. Interestingly, Cox inhibition is pH-dependent *(Nicholls and Kim, 1982)*. It is actually suggested that acidosis shifts the balance of protonated (H_2S) and deprotonated (HS^-) forms in solution, and these two forms may have a different affinity for Cox *(Szabo et al., 2014)*. This characteristic is of great importance as it suggests enhanced inhibitory effect in condition/sites with local acidosis.

2.2. Stimulatory effects of H_2S on mitochondrial respiration

Besides the ability of circulating oxidizers to consume and eliminate H_2S, the main pathway of H_2S catabolism operates in mitochondria. At nanomolar to low micromolar concentrations, sulfide can transfer electrons to coenzyme Q, which then follow the normal oxidative route to complex III and IV, activating mitochondrial respiration and ATP production (figure 8) *(Goubern et al., 2007; Modis et al., 2013)*. Initial work utilizing colonocytes described that electron transfer from H_2S to mitochondrial ETC implicates a sulfide oxidation unit (SOU) that consists of three enzymes: a sulfide quinone reductase, a dioxygenase and a sulfur transferase *(Goubern et al., 2007)*. Oxidation of H_2S by SOU produces thiosulfate ($H_2S_2O_3$) by consuming an additional molecule of O_2.

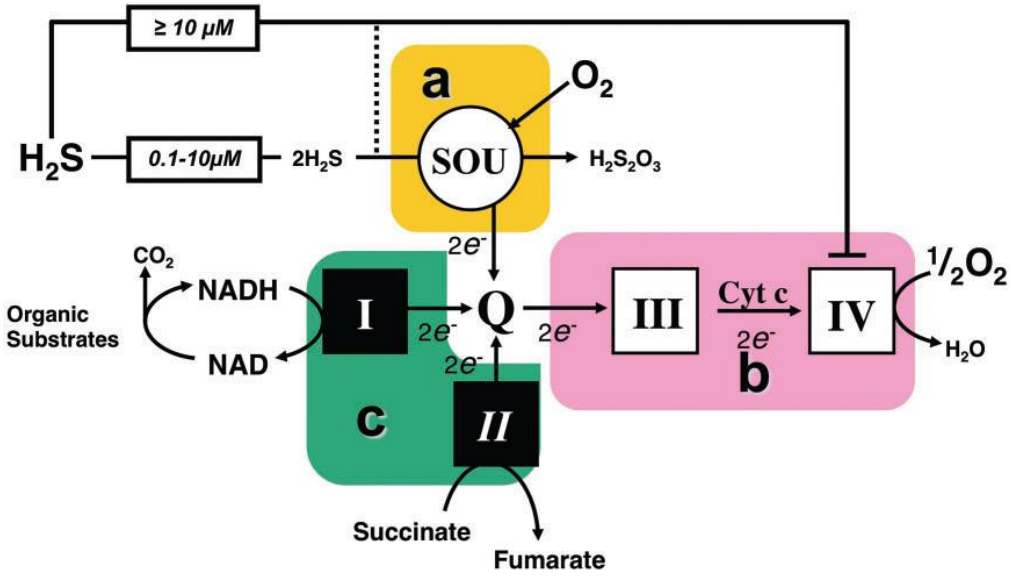

Figure 8: Dual effects of H_2S on the mitochondrial electron transport chain. The sulfide oxidation unit (SOU), which oxidizes two molecules of sulfide and uses one O_2 molecule, releases thiosulfate and reduces quinone Q (*yellow box* **a**). Complex III accepts electrons from ubiquinone and drives them through complex IV (*pink box* **b**). Block c (*green box*) is constituted by the other enzymatic reactions reducing ubiquinone. Complex IV is the target of sulfide inhibition, becoming significant at concentrations of 10 µM and above, whereas SOU operates well below 10 µM concentration. Szabo *et al*., 2014.

This nature of H_2S serving as an ETC substrate is functionally comparable to TCA cycle-derived electron donors (NADH or $FADH_2$), although the yield of sulfide in terms of electrons to be used by the ETC is relatively low (1 molecule of sulfide provides 1 electron, whereas NADH can donate 2 electrons and $FADH_2$ 2 electrons). Moreover, because of the additional O_2 consumption implicated in the sulfide oxidation by SOU, the yield in energy per O_2 consumed is low compared to NADH and $FADH_2$ *(Szabo et al., 2014)*. This process may have been the primordial version of energetic metabolism of deep-see living unicellular species that evolved billions of years ago *(MacLeod et al., 1994)*.

3. Biological functions

H_2S has specific physiological functions in different systems. In the nervous system, H_2S exerts multifaceted effects by activating N-methyl-D-aspartate (NMDA) receptors *(Kimura, 2002)* and by directly increasing glutamate secretion *(Garcia-Bereguiain et al., 2008)*. In the endocrine system, H_2S controls pancreatic insulin metabolism as well as glucose homeostasis *(Ali et al., 2007)*. In the gastro-intestinal system, sulfate-reducing bacteria (SRB) present in the lumen of mice and humans can produce 0.2-3.4 mM H_2S *(Blachier et al., 2010)*, but the efficient sulfide oxidation to thiosulfate and sulfate by the colonic mucosa allows protection against high concentrations of H_2S *(Goubern et al., 2007)*. In the colon, H_2S has been reported to have both pro and anti-inflammatory effects, to mediate smooth muscle relaxation and epithelial secretory function, and to modulate nociception *(Medani et al., 2011)*.

The most described physiological effects of H_2S are in the vascular system. H_2S-induced vasorelaxation has been demonstrated in numerous types of blood vessels from different species *(Wang, 2012)*. H_2S regulates the vascular tone mainly by opening ATP-sensitive potassium (K_{ATP}) channels *(Cheng et al., 2004)*. As illustrated in figure 9, K_{ATP} activation leads to hyperpolarization of vascular smooth muscle cells, causing vasodilation by reducing voltage-gated Ca^{2+} influx. K_{ATP} channels are composed of pore-forming (Kir 6.X)

and sulfonylurea receptor (SUR) subunits. Compiling evidence suggests that SUR receptors are the sites of modulation by H_2S: K^+ currents generated by expression of Kir subunits in the absence of SUR receptors are insensitive to H_2S *(Jiang et al., 2010)*. Earlier data indicating that H_2S increases the open probability of K_{ATP} channels in excised membrane patches (independently of ATP levels) supports a direct mechanism of H_2S action *(Yang et al., 2005)*. Furthermore, alkylation or oxidation of cysteine residues located on the extracellular face of the SUR1 protein prevents regulation by H_2S *(Jiang et al., 2010)*.

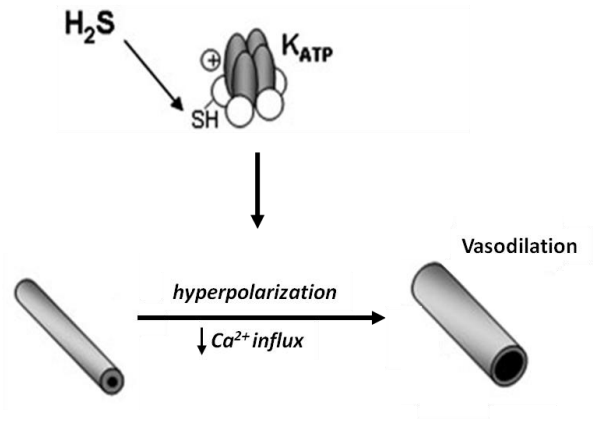

Figure 9: The K_{ATP} channel comprises four channel subunits (Kir 6.X) associated to four sulfonylurea receptors (SUR). The channel is activated by H_2S through modification of SUR cysteine residues. Vascular K_{ATP} channels opening leads to hyperpolarization and relaxation of vascular smooth muscles, which induces vasorelaxation and lowers the blood pressure. Adapted from *(Peers et al., 2012)*.

A NO-dependent vasoactive mechanism has also been proposed, conferring a dual modulation of vascular tension to H_2S. Indeed, several studies reported that H_2S may promote *(Hosoki et al., 1997; Predmore et al., 2011)* or inhibit *(Geng et al., 2007; Kubo et al., 2007)* NO production, leading to either a synergistic or an antagonistic relaxation of blood vessels.

Intriguingly, different vascular tissues show different sensitivities to H_2S. For example, it has been reported that H_2S relaxes small mesenteric arteries six times more efficiently than the aorta *(Cheng et al., 2004)*. The mechanisms for the differential vasodilatory effects of H_2S may be related to the tissue-specific distribution of its molecular targets. In addition, different type of blood vessels face different sheer stress levels, possess different cellular and connective components, and/or have a different stiffness, which can further affect the biological response to H_2S *(Wang, 2012)*. Finally, the O_2-dependent sensitivity of blood vessels to H_2S must be highlighted. Indeed, K_{ATP} subunits expression in human myocardium is inversely correlated with venous pO_2 and culturing myocytes under hypoxic conditions results in HIF-1-dependent upregulation of the K_{ATP} channel *(Raeis et al., 2010)*. It was also reported that H_2S induces vasorelaxation much faster in a hypoxic compared to normoxic environment *(Koenitzer et al., 2007)*.

4. Pre-clinical studies on the role of H_2S in cancer

Many studies have focused on the potential role of H_2S in colon cancer development. Analysis of human colon cancer biopsies and patient-matched normal mucosa revealed the selective upregulation of H_2S-producing enzyme CBS, resulting in an increased rate of H_2S production in cancer cells *(Szabo and Hellmich, 2013)*. Using small hairpin ribonucleic acid (shRNA) -mediated silencing or pharmacological inhibition of CBS, authors of this study demonstrated that H_2S promotes HCT116 human colon cancer cells proliferation because it stimulates bioenergetics (both OXPHOS and glycolysis). Effect of H_2S on glycolysis was attributed to an increase in the catalytic activity of the enzyme GAPDH via sulfhydratation *(Mustafa et al., 2009)*. Treatment of nude mice with a specific CBS inhibitor attenuated the growth of patient-derived colon cancer xenografts. It was also suggested that CBS-derived H_2S can promote vasorelaxation and angiogenesis, thereby providing tumors with blood and nutrients and increasing tumor invasion capacity *(Szabo and Hellmich, 2013)*. A summary of the proposed mechanisms for H_2S induced colon cancer growth is presented in figure 10.

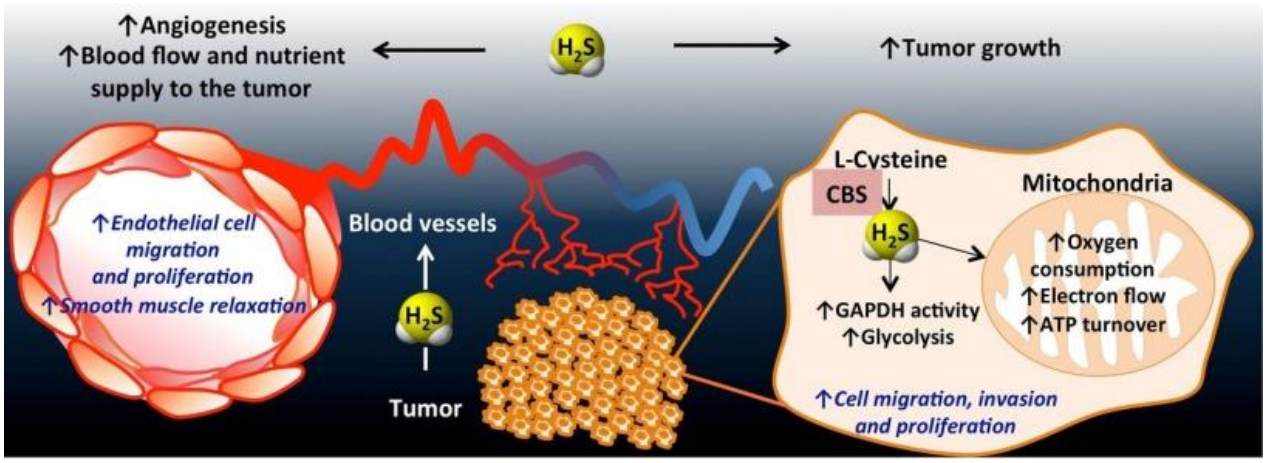

Figure 10: Overexpression of CBS in cancer cells leads to H_2S overproduction. H_2S acts as an electron donor and stimulates mitochondrial electron transport. In addition, H_2S increases glycolysis by increasing the catalytic activity of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). These autocrine effects have been suggested to increase proliferation, migration and invasion. By diffusing into the surrounding tissue, H_2S further stimulates angiogenesis and acts as a vasodilator, thereby also promoting the supply of blood and nutrients to the tumor. Zsabo and Hellmich, 2013.

In another study, exogenous administration of a fast H_2S-releasing inorganic donor (NaHS) also induced the proliferation of HCT116 human colon cancer cells in a concentration-dependent manner, with the optimal proliferative concentration identified to be 200 µM. Authors suggested that this effect might be mediated by increasing Akt and ERK phosphorylation and by inhibiting the cyclin-dependent kinase inhibitor P21, resulting in an increased proportion of cells in S phase *(Cai et al., 2010)*.

Unexpectedly, Wu *et al*. *(Wu et al., 2012)* reported opposite results regarding the effect of H_2S on colon cancer cell proliferation. In the same cancer cell line (HCT116) but with higher NaHS concentrations (400 µM-1 mM), H_2S inhibited cell proliferation through activation of the AMPK pathway. Conflicting conclusions are probably related to the known

bell-shaped dose-response curve of H_2S, where lower levels of H_2S exert physiological and cytoprotective effects and higher local H_2S concentrations promotes cytotoxicity *(Wang, 2012)*.

A few years ago, a group characterized a novel, water-soluble, slow-releasing H_2S compound (GYY4137), and evaluated its use as an anticancer agent in two studies. In the first study, they showed that compared to NaHS that releases H_2S within minutes, GYY4137 releases H_2S much slower, with H_2S concentrations remaining higher than baseline for up to 7 days in the culture medium (figure 11) *(Lee et al., 2011)*.

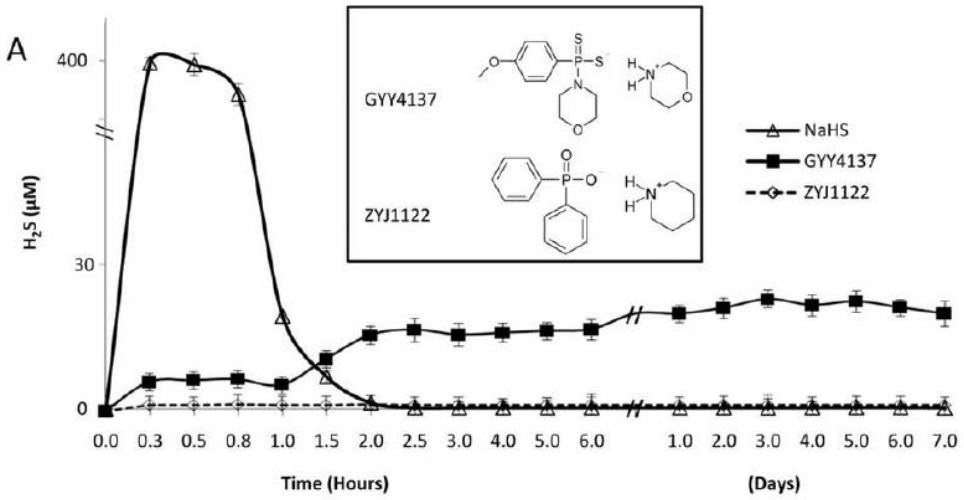

Figure 11: Differential H_2S-releasing kinetics of NaHS and GYY4137. Concentrations of H_2S were assessed spectrophotometrically using a methylene blue-based method. ZYJ1122 is a control for GYY4137 lacking sulfur and unable to produce H_2S. Lee *et al*., 2011.

In the first study, the single exposure of MCF-7 (breast adenocarcinoma), MV4-11 (acute promyelocytic leukemia) and HL-60 (myelomonocytic leukemia) tumor cells to 400 μM NaHS did not increase or decrease cancer cell proliferation after 5 days, whereas GYY4137 caused a concentration-dependent reduction in cancer cell proliferation and survival, with cell cycle arrest in the G2/M phase and promotion of apoptosis *(Lee et al.,*

2011). More recent data indicate that continuous and low exposure to H_2S was necessary to selectively target cancer cells. Replacement of NaHS (5-20 µM) every 2 hours for 5 days significantly decreased cell viability and had a more important effect on malignant compared to non-malignant cells *(Lee et al., 2014)*. These authors further suggested that H_2S had combined effects on glycolysis and pHi regulators. It was proposed that H_2S concomitantly increases metabolic acid production through glycolysis and impairs pHi regulation, resulting in cytotoxic intracellular acidification *(Lee et al., 2014)* (figure 12).

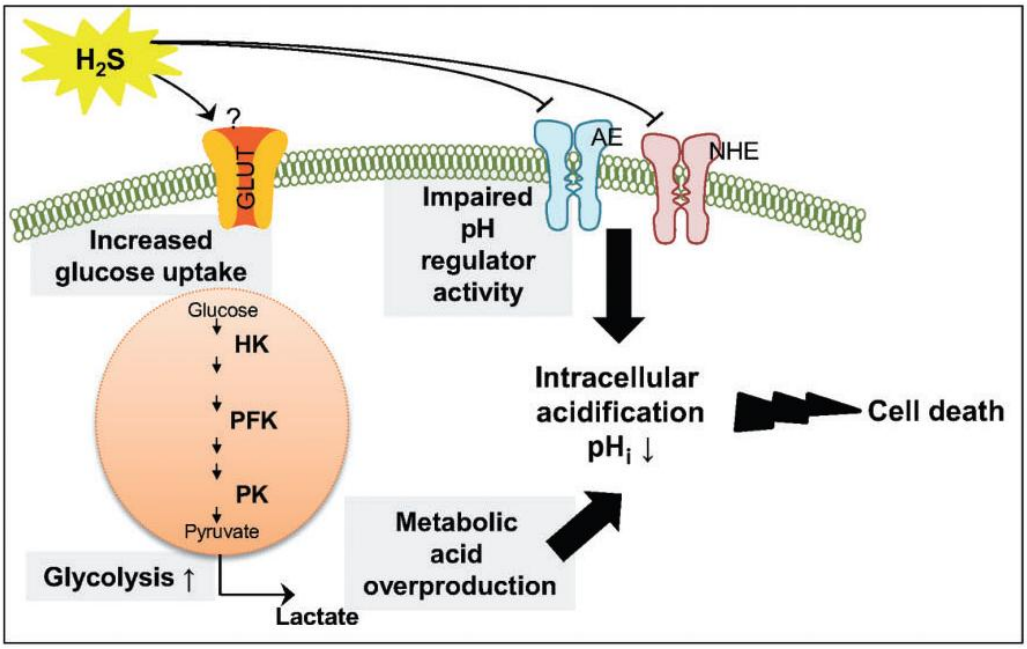

Figure 12: H_2S promotes an influx of glucose and triggers enhanced glycolysis in cancer cells. Overproduction of lactate coupled with an impaired activity of pH regulators results in the accumulation of acid and in a reduction of pHi. pHi then serves as a stimulus to induce cancer cell death. AE: anion exchanger, GLUT: glucose transporter, HK: hexokinase, NHE: sodium/proton exchanger, PFK: phosphofructokinase, PK: pyruvate kinase. Lee *et al.,* 2014.

Finally, although the anticancer properties of NaHS have not been studied *in vivo*, GYY4137 administered daily to nude mice caused a dose-dependent reduction in the progression of two human leukemia tumor models *(Lee et al., 2011)*.

5. H_2S in combination with radiotherapy

In 2011, Zhang and colleagues investigated whether H_2S could influence hypoxia-induced radioresistance *in vitro (Zhang et al., 2011).* Experiments showed that, when hypoxic human hepatoma HepG2 cells were treated with 50 or 100 μM NaHS before irradiation, cells became more radioresistant so that survival was enhanced. Radioresistance was limited to lower concentrations of NaHS (25-100 μM). When the concentration of NaHS exceeded 200 μM, its protective effect was lost and the treatment became cytotoxic. This phenomenon is consistent with the fact that H_2S has opposite actions depending on its concentration and the time of application. Authors of the study hypothesized that H_2S may induce the radioresistance of hypoxic tumor cells via activation of K^+_{ATP} channels and inhibition of Ca^{2+} flux. This study suggested that H_2S (derived from NaHS) may confer intrinsic radioresistant properties to cancer cells.

Chapter 2 Aims of the thesis

This thesis pursued a dual objective. In one part, the aim was to experimentally validate the hypothesis suggesting that rewiring glucose metabolism benefits to both energetic and biosynthetic demands of cancer cells. Indeed, enhanced glycolysis potentially allows fast ATP production and provides carbon intermediates that can be shunted to anabolic pathways. For the purpose, we compared the metabolic profile of different cancer cell lines and analyzed their intracellular ATP concentration and DNA synthesis rate. We paid attention to how enhanced glycolysis promotes cancer cell proliferation, as it could help to identify new mechanisms by which glycolysis-targeting drugs impair tumor growth. The pentose phosphate pathway (PPP) was specifically analyzed because it uses glycolytic intermediates to supply cells with nucleotide precursors and NADPH, a crucial reductant in anabolic processes. Furthermore, we aimed to study the therapeutic relevance of these findings. Dichloroacetate (DCA) was used because it had already demonstrated interesting anticancer properties *in vitro* and *in vivo,* and is currently tested in clinical trials. However, its mechanism of action, which globally consists in induction of apoptosis, has recently been questioned. We thus analyzed whether DCA interferes with the PPP in order to propose a new mechanism by which this promising molecule impairs tumor progression.

The second part of this work focused on mitochondria as target for cancer therapy, especially in combination with radiotherapy (RT). Experimental evidence suggested that inhibition of O_2 consumption may be the most effective way to reduce hypoxia, a major contributor to RT failure in the treatment of solid tumors. Unfortunately, inhibition of tumor cell O_2 consumption in combination with RT has attracted only little interest in the clinics, as compared to provascular approaches. In this context, we hypothesized that hydrogen sulfide (H_2S), the most recently identified gasotransmitter in mammals, could radiosensitize solid

tumors. Indeed, H_2S is known to primarily target the mitochondrial ETC. Moreover, it is implicated in the vasorelaxation of blood vessels, which could also improve tumor oxygenation. We therefore used sodium hydrosulfide (NaHS) as a H_2S donor, and questioned whether it could potentially alleviate tumor hypoxia and consequently radiosensitize experimental tumors by an oxygen effect. To help understand the existing conflictual data pertaining to the implication of H_2S in cancer progression, we also analyzed the effects of NaHS in monotherapy *in vitro* and *in vivo.*

Chapter 3 Glycolysis drives cancer cell proliferation

Inhibition of the pentose phosphate pathway by dichloroacetate unravels a missing link between aerobic glycolysis and cancer cell proliferation

Géraldine De Preter, Marie-Aline Neveu, Pierre Danhier, Lucie Brisson, Valéry L. Payen, Paolo E. Porporato, Bénédicte F. Jordan, Pierre Sonveaux and Bernard Gallez. Oncotarget. 2015; DOI: 10.18632/oncotarget.6272

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Inhibition of the pentose phosphate pathway by dichloroacetate unravels a missing link between aerobic glycolysis and cancer cell proliferation

Géraldine De Preter[1], Marie-Aline Neveu[1], Pierre Danhier[1], Lucie Brisson[2], Valéry L. Payen[2], Paolo E. Porporato[2], Bénédicte F. Jordan[1], Pierre Sonveaux[2] and Bernard Gallez[1]

[1] Louvain Drug Research Institute (LDRI), Biomedical Magnetic Resonance Research Group, Université catholique de Louvain (UCL), Brussels, Belgium

[2] Institut de Recherche Expérimentale et Clinique (IREC), Pole of Pharmacology, Université catholique de Louvain (UCL), Brussels, Belgium

Correspondence to: *Bernard Gallez,* ***email:*** *bernard.gallez@uclouvain.be*

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ABSTRACT

Glucose fermentation through glycolysis even in the presence of oxygen (Warburg effect) is a common feature of cancer cells increasingly considered as an enticing target in clinical development. This study aimed to analyze the link between metabolism, energy stores and proliferation rates in cancer cells. We found that cell proliferation, evaluated by DNA synthesis quantification, is correlated to glycolytic efficiency in six cancer cell lines as well as in isogenic cancer cell lines. To further investigate the link between glycolysis and proliferation, a pharmacological inhibitior of the pentose phosphate pathway (PPP) was used. We demonstrated that reduction of PPP activity decreases cancer cells proliferation, with a profound effect in Warburg-phenotype cancer cells. The crucial role of the PPP in sustaining cancer cells proliferation was confirmed using siRNAs against glucose-6-phosphate dehydrogenase, the first and rate-limiting enzyme of the PPP. In addition, we found that dichloroacetate (DCA), a new clinically tested compound, induced a switch of glycolytic cancer cells to a more oxidative phenotype and decreased proliferation. By demonstrating that DCA decreased the activity of the PPP, we provide a new mechanism by which DCA controls cancer cells proliferation.

INTRODUCTION

These last few years, metabolism has generated tremendous interest in the field of cancer research. Many studies focused on the various metabolic profiles of different tumors [1-3] because metabolic plasticity is involved in cancer progression, drug resistance and metastasis [4-6]. In normal cells, glycolysis is coupled to oxidative phosphorylation (OXPHOS) to optimally synthesize intracellular ATP from glucose [7]. However, many cancer cells undergo a fundamental metabolic transformation, the "glycolytic switch", by which glycolysis is uncoupled from respiration and rewired to lactic fermentation, thus becoming the primary source of cell ATP production. Switching to a glycolytic metabolism primarily occurs under hypoxia as a rescue mechanism for energy production. However, some cancer cells further adopt a particular glycolytic phenotype, first described by Warburg [8] and coined 'aerobic glycolysis' [9]. The biological rationale behind the Warburg phenotype remains controversial, but it has been recently proposed that proliferating cancer cells enhance glycolysis because it benefits both bioenergetics and biosynthesis [4, 10]. Indeed, a glycolytic metabolism potentially allows fast ATP production and provides carbon intermediates that can be directed to branched biosynthetic pathways, enabling a faster expansion of the cellular biomass. Convincingly, mutations occurring in signaling pathways regulating both cellular biosynthesis and aerobic glycolysis, such as the PI3K/Akt/mTOR pathway, are the most prevalent

class of mutations in human tumors [11]. However, experimental evidence linking aerobic glycolysis to cancer cell proliferation is lacking, and the selective advantage provided by this phenotype is not entirely clear. The aim of the present study was to elucidate the coupling between metabolism, energy supply and cell proliferation in various human and murine cancer cells. Metabolic switches were induced to provide evidence that bioenergetics, and more particularly glycolysis, directly drives cancer cell proliferation. In this line, we found a new therapeutic mechanism of dichloroacetate (DCA), an activator of the mitochondrial oxidation of glucose currently investigated in clinical studies [12]. DCA inhibited the pentose phosphate pathway (PPP), a pivotal biosynthetic pathway branched to glycolysis. We report that the PPP bridges the gap between a glycolytic metabolism and cancer cell proliferation.

RESULTS

Glycolytic efficiency is positively linked to proliferation but not to ATP levels in cancer cells

A screening of the metabolic activities of six cancer cell lines was first performed in order to investigate the link between glucose metabolism, ATP stores and proliferation capacity. OXPHOS, evaluated by the oligomycine-sensitive oxygen consumption rate (mitoOCR), and glycolytic efficiency, measured by the lactate produced to glucose consumed (mol/mol) ratio [13], were assessed separately on cells incubated 24 hours in a culture medium containing only glucose as energetic fuel. To investigate the influence of the metabolic status on

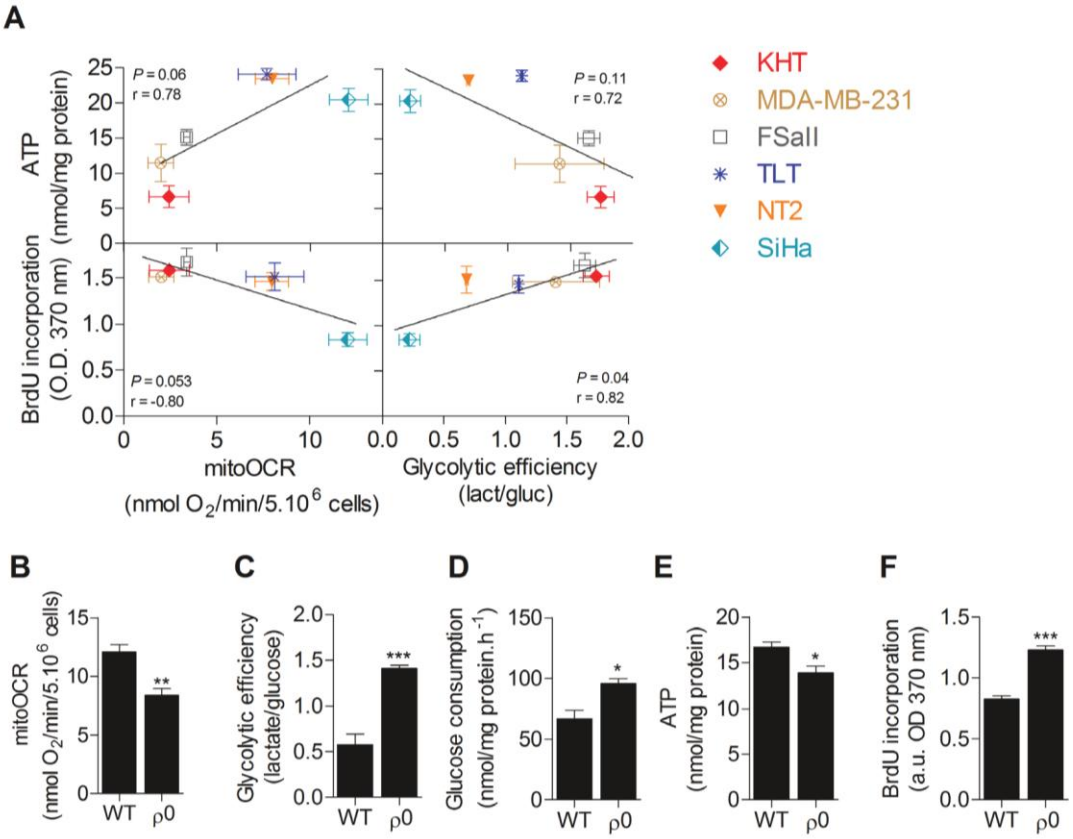

Figure 1: Glycolytic efficiency is positively linked to proliferation but not to ATP levels in cancer cells. A. Correlation plots between metabolic parameters (glycolytic efficiency and mitochondrial oxygen consumption rate [mitoOCR]), intracellular ATP content and proliferation of six human and murine cancer cell lines. Measurements were performed after 24 h incubation in the presence of a culture medium containing only glucose as energetic fuel. The mitoOCR was determined by the oligomycin-sensitive OCR of viable whole cells and the glycolytic efficiency (glucose consumption/lactate production ratio) was measured from cells supernatant. Total ATP was quantified from lysed cells and normalized to protein content. Proliferation rates were analyzed by the incorporation of a nucleotid analog (5-bromo-2'-deoxyuridine [BrdU]). A significant correlation was found between glycolytic efficiency and proliferation (p-value = 0.04, Pearson r = 0.82). Non-significant correlations were found between mitoOCR and ATP content (p-value = 0.06, Pearson r = 0.78), mitoOCR and proliferation (p-value = 0.053, Pearson r = -0.80) and between glycolytic efficiency and ATP content (p-value = 0.11, Pearson r = 0.72). **B.-F.** Comparison of the metabolic profile (B-C-D), intracellular ATP content **E.** and proliferation **F.** between wild-type (WT) and mitochondria-depleted (ρ0) isogenic SiHa cancer cells. Two-sided t test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Results are expressed as means ± SEM.

cellular energy stores, total intracellular ATP pool was also measured. No significant correlation was found between intracellular ATP content and the metabolic parameters. However, we found that cell proliferation evaluated by DNA synthesis was significantly correlated to glycolytic efficiency (Figure 1A), where increased glycolysis was associated with an increased proliferation capacity, and inversely. No significant correlation was observed between proliferation capacity and mitochondrial respiration. These data suggest that glycolysis is potentially the key energetic pathway supporting cancer cell proliferation. It is currently hypothesized that this pathway supports cell proliferation predominantly by supplying precursors for biosynthetic pathways, rather than by ATP production [10, 11]. Supporting that ATP supply is not the main limiting factor for cell proliferation, no correlation between ATP content and DNA synthesis was found in the cell lines (Supplemental Figure S1). To confirm that glycolysis promotes cancer cell proliferation and exclude metabolism-independent influences inherent to different genotypes, the proliferation capacity of human wild-type (WT) oxidative SiHa cervical cancer cells was compared to that of SiHa with partial mitochondrial depletion (SiHa ρ0) [14, 15]. SiHa ρ0 cancer cells exhibited a glycolytic phenotype with a ~40% decrease in mitoOCR (Figure 1B), a ~2.5 fold increase in glycolytic efficiency (Figure 1C) and a ~50% increase in glucose consumption (Figure 1D). The net ~20% decrease in total ATP pool in SiHa ρ0 cells confirmed that enhanced glycolysis was not sufficient to maintain ATP stores (Figure 1E). By performing DNA synthesis quantification, we found that enhanced aerobic glycolysis promotes cell proliferation, as the glycolytic switch in SiHa ρ0 cells was associated with a ~45% increase in cell proliferation (Figure 1F). Viability assays revealed no difference in viable cell number 24 h after cells were incubated in the experimental culture medium (Supplemental Figure S2), likely because DNA synthesis quantification detects early changes in the proliferation rate of the cells.

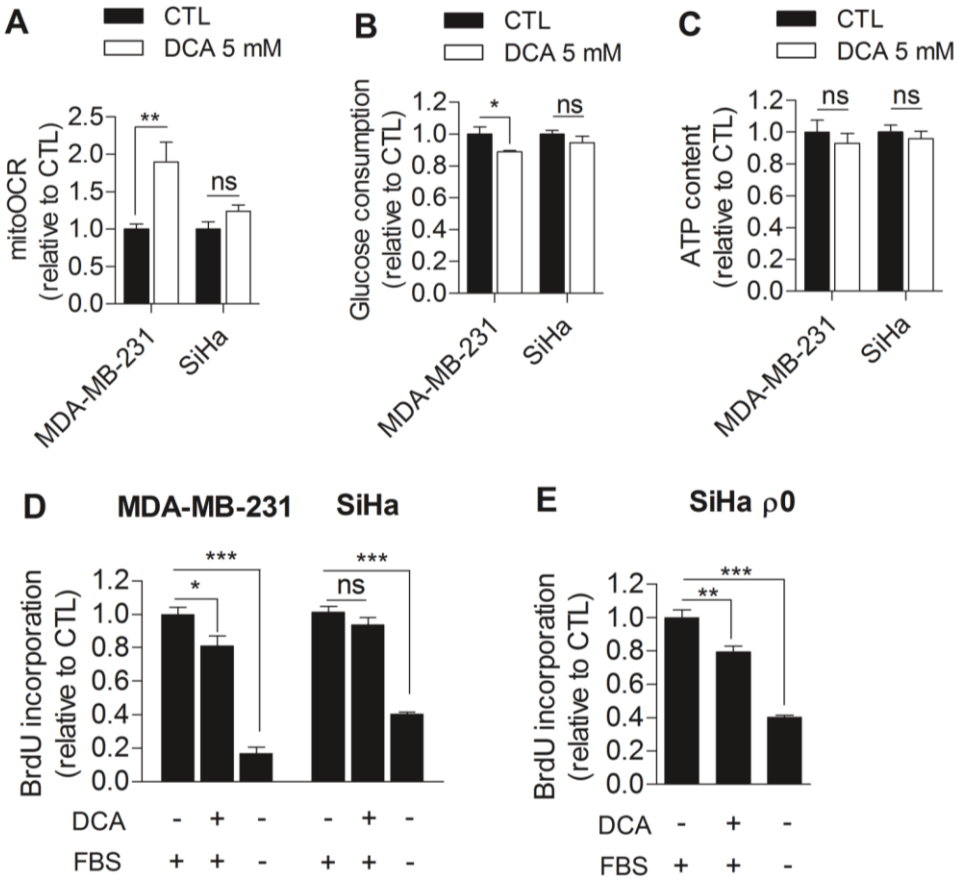

Figure 2: DCA significantly influences the metabolism and proliferation of glycolytic but not oxidative cancer cells. **A.** Mitochondrial oxygen consumption rate, **B.** glucose consumption, **C.** intracellular ATP content and **D.** proliferation of MDA-MB-231 (glycolytic) and SiHa (oxidative) human cancer cells after 48 h dichloroacetate (DCA) 5 mM treatment. **E.** Proliferation of mitochondria-depleted (ρ0) SiHa cancer cells after 48 hours DCA 5 mM treatment. Medium containing no FBS was used as positive control in proliferation experiments. Two-sided *t* test **A.-C.** or one-way ANOVA with Bonferroni post-hoc test **D.-E.** $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, ns, not significant. Results are expressed as the relative change from control cells and as means ± SEM.

Glycolysis inhibition by DCA impairs cancer cell proliferation

Based on our observations, we further investigated whether glycolysis inhibition could directly impair cancer cell proliferation. For the purpose, MDA-MB-231 human breast cancer cells (Warburg phenotype, Figure 1A) and SiHa human cervical cancer cells (oxidative phenotype, Figure 1A) were treated with dichloroacetate (DCA), a pyruvate dehydrogenase kinase (PDK) inhibitor that enhances the oxidative activity of cells by activating pyruvate dehydrogenase (PDH), the gate-keeping enzyme of glucose oxidation in mitochondria [16]. To date, the promising therapeutic effect of DCA on cancer cells is globally attributed to a normalization of the hyperpolarized mitochondrial membrane potential characterizing cancer cells and to re-sensitization to apoptosis [17]. Here, we postulated that DCA also controls tumor proliferation by inhibiting glycolysis. To test this hypothesis, glycolytic MDA-MB-231 and oxidative SiHa cancer cells were treated with 5 mM DCA for 48 h, and the effects of the treatment on metabolism and proliferation were assessed. Compared to vehicle-treated cells, DCA induced a switch of glycolytic MDA-MB-231 cancer cells to a more oxidative phenotype as evidenced by an increase in mitoOCR (Figure 2A) and a decrease in glucose consumption (Figure 2B). The decrease in glycolytic activity observed in this experiment is consistent with another recent study [18] and is likely induced by the Pasteur Effect [4, 19] to maintain ATP homeostasis in the cells (Figure 2C). We also observed that glycolysis inhibition by DCA was associated with a decreased proliferation rate of MDA-MB-231 cancer cells (Figure 2D). Supporting that glycolysis inhibition impairs proliferation in this cell line, 2-Deoxy-D-glucose-treated MDA-MB-231 cancer cells also exhibited a decreased proliferation rate (Supplemental Figure S3).

On the other hand, DCA did not significantly alter

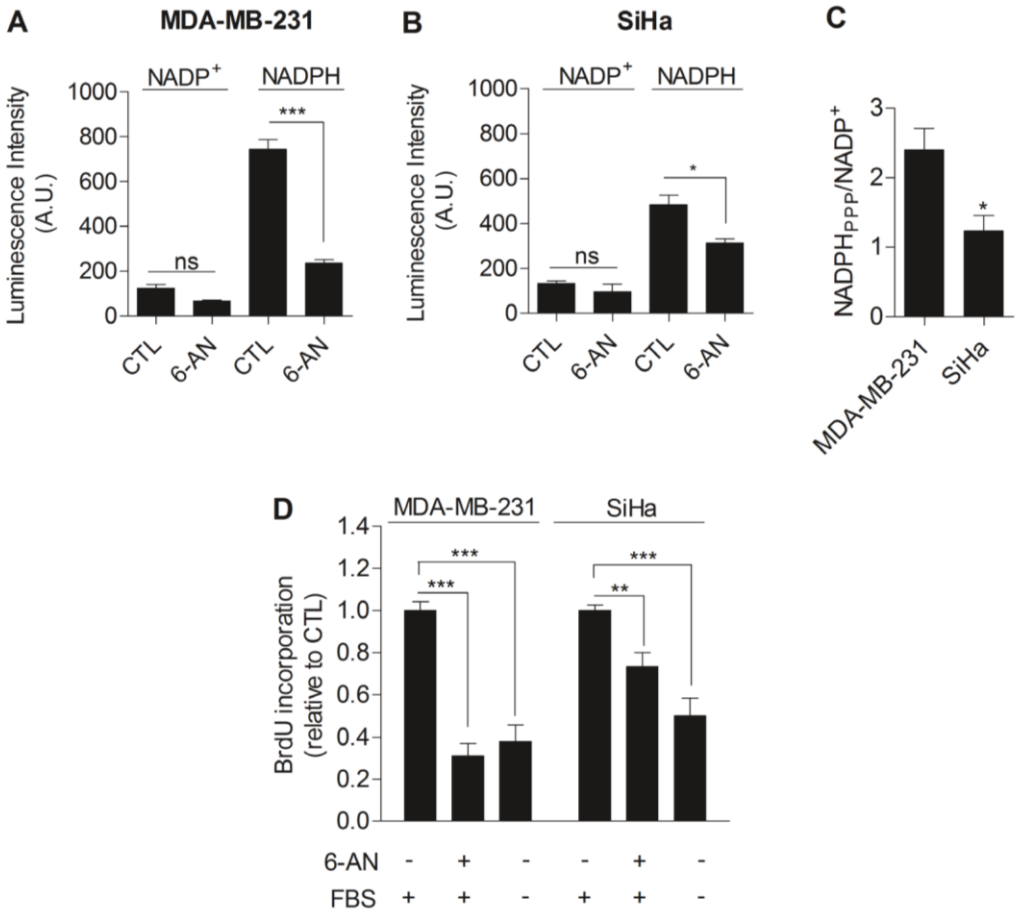

Figure 3: The PPP differentially supports proliferation in glycolytic and oxidative cancer cells. Intracellular $NADP^+$ and NADPH levels measured individually in viable glycolytic MDA-MB-231 **A.** and oxidative SiHa **B.** cancer cells. 6-aminonicotinamide (6-AN), a specific inhibitor of the PPP, was used (100 μM, 48 h treatment) to highlight NADPH production from the PPP ($NADPH_{ppp}$). **C.** $NADPH_{PPP}/NADP^+$ ratios in MDA-MB-231 and SiHa cancer cells. **D.** Proliferation measured by the incorporation of BrdU in MDA-MB-231 and SiHa cancer cells after exposure to 6-AN (100 μM, 48 h treatment). Medium containing no FBS was used as positive control in proliferation experiments. Two-sided *t* test **A.-C.** or one-way ANOVA with Bonferroni post-hoc test **D.**. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, ns, not significant. Results are expressed as means ± SEM.

the metabolic activities of oxidative SiHa cancer cells (Figure 2A-2C) and had no significant effects on SiHa proliferation (Figure 2D). Short-term (1 hour) lactate production measurements showed that DCA was indeed more effective in the glycolytic cancer cell line than in the oxidative one (Supplemental Figure S4). To investigate whether the metabolic profile determine the response to DCA, the proliferation capacity of glycolytic SiHa ρ0 cancer cells was also analyzed after DCA treatment. We found a significant decrease in DNA synthesis in this cell line (Figure 2E), an effect that was not observed in SiHa WT. Furthermore, the same number of viable MDA-MB-231 and SiHa ρ0 cancer cells were measured 48 h after treatment with vehicle or DCA (Supplemental Figure S5 A-B), showing that the effects of DCA on metabolic functions and proliferation rates are not due to cell mortality.

Glycolysis controls cancer cell proliferation through the pentose phosphate pathway

Taken together, our data provided compiling experimental evidence that glycolysis *per se* is involved in the control of cell proliferation. The mechanism linking glycolysis and proliferation still remained to be established. We postulated that the pentose phosphate pathway (PPP) could link glycolysis to proliferation, as the PPP uses glycolytic intermediates to supply cells with nucleotides and NADPH, a crucial reductant in anabolic processes [20]. To specifically determine NADPH produced from the PPP ($NADPH_{ppp}$), cells were treated with 6-aminonicotinamide (6-AN), a specific inhibitor of the PPP [21, 22]. We found that the contribution of $NADPH_{ppp}$ to the total NADPH pool ($NADPH_{tot}$) was more predominant in MDA-MB-231 cancer cells than in SiHa cancer cells, as evidenced by a major decrease in $NADPH_{tot}$ level following 6-AN treatment in glycolytic MDA-MB-231 cells (Figure 3A). More limited effects were seen in SiHa oxidative cells (Figure 3B). Measurements also revealed higher $NADPH_{ppp}$/

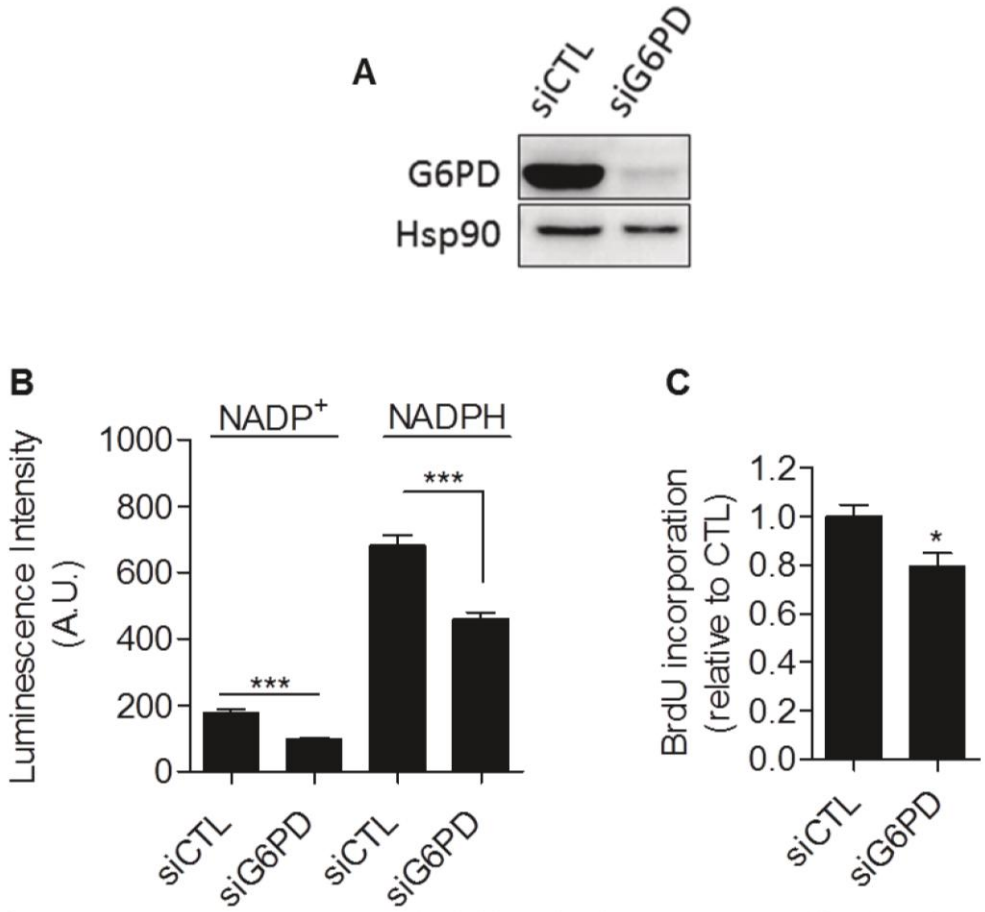

Figure 4: Glucose-6-phosphate dehydrogenase inhibition with siRNA reduces proliferation of glycolytic cancer cells. MDA-MB-231 cancer cells were transfected with siRNAs targeting G6PD (siG6PD) or non-targeting siRNAs (siCTL). 48 hours after transfection, cells were subjected to **A.** immunoblot analysis using Hsp90 as loading control, **B.** $NADP^+$ and NADPH levels quantification and **C.** DNA synthesis measurement. Two-sided *t* test. $*p < 0.05$, $***p < 0.001$. Results are expressed as means ± SEM.

$NADP^+$ ratio in MDA-MB-231 cancer cells, highlighting a higher PPP flux in this glycolytic cell line compared to SiHa (Figure 3C). To verify that the PPP is involved in the control of cancer cells proliferation, DNA synthesis in 6-AN-treated and non-treated cells was evaluated. We observed that the proliferation capacity of cancer cells was impaired when the PPP was inhibited (Figure 3D). Interestingly, a stronger effect was evidenced in glycolytic MDA-MB-231 (~70 % decrease in DNA synthesis rate) compared to oxidative SiHa (~25 % decrease in DNA synthesis rate) cancer cells (Figure 3D). Importantly, SiHa ρ0 were more sensitive than SiHa WT to 6-AN as a ~40 % decrease in DNA synthesis was found (Supplemental Figure S6). Owing to the potential off-target effects of pharmacological inhibitors, we complemented our 6-AN studies using small interfering RNAs (siRNAs) targeting glucose-6-phosphate dehydrogenase (G6PD), the first and rate-limiting enzyme of the PPP [23]. In MDA-MB-231 transfected cells, we confirmed silencing of G6PD by immunoblotting (Figure 4A) and demonstrated that, like 6-AN, inhibition of G6PD decreased PPP activity (Figure 4B) and DNA synthesis (Figure 4C). These results point out the predominant contribution of the PPP in sustaining the proliferation of Warburg-phenotype cancer cells.

DCA inhibits the pentose phosphate pathway

Finally, we investigated whether DCA could inhibit the PPP, which would explain the link between glycolysis inhibition and the decreased proliferation rate of DCA-treated cancer cells. As shown in Figure 5, we observed that DCA (5 mM, 48 h) significantly decreased $NADPH_{tot}$ level in MDA-MB-231 cancer cells (Figure 5A). Furthermore, while no additional decrease in $NADPH_{tot}$ was obtained when cells were exposed to DCA and 6-AN together (Figure 5A), we showed that DCA specifically decreased $NADPH_{PPP}$. Similar results were found using molecular inhibition of G6PD (Figure 5B). These findings demonstrate the implication of the PPP in the effects of DCA on glycolytic cancer cells.

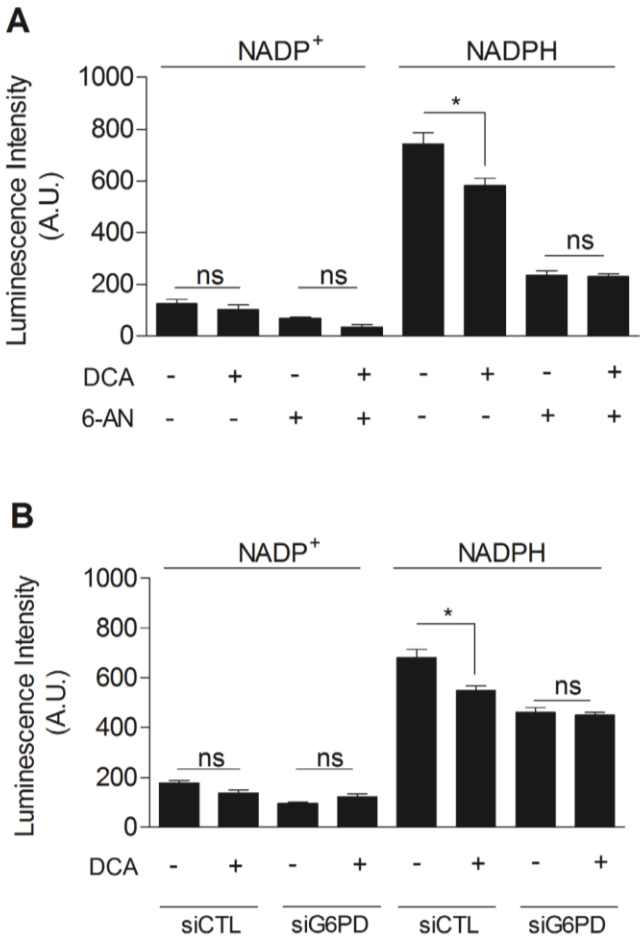

Figure 5: DCA decreases PPP activity. A. Intracellular $NADP^+$ and NADPH levels measured in MDA-MB-231 cancer cells treated with ± DCA (5 mM, 48 h) and ± 6-AN (100 µM, 48 h). **B.** Intracellular $NADP^+$ and NADPH levels measured in MDA-MB-231 cancer cells transfected with siRNAs against G6PD (siG6PD) or non-targeting siRNAs (siCTL) and treated with ± DCA. One-way ANOVA with Bonferroni post-hoc test. *$p < 0.05$, ns, not significant. Results are expressed as means ± SEM.

DISCUSSION

This study emphasizes the predominant role of the pentose phosphate pathway in cancer biology. The Warburg effect (the fermentation of glucose to lactate in the presence of a physiological O_2 concentration) observed in numerous cancer cells has been proposed to meet both energetic (ATP) and biosynthetic demands [4, 10]. In this study, we showed that enhanced aerobic glycolysis was not sufficient to maintain ATP levels in cancer cells. Hence, low intracellular ATP level was associated with increased glycolysis. Moreover, the glycolytic switch induced in mitochondria-deficient cancer cells was not able to provide adequate ATP supply as compared to wild-type cells. It is actually suggested that, bioenergetically speaking, a glycolytic metabolism is less efficient than an oxidative one, but that glycolysis may be advantageous to rapidly produce ATP to meet short-timescale energy demands [24]. Our findings also corroborates that Warburg-phenotype cancer cells develop several adaptations to

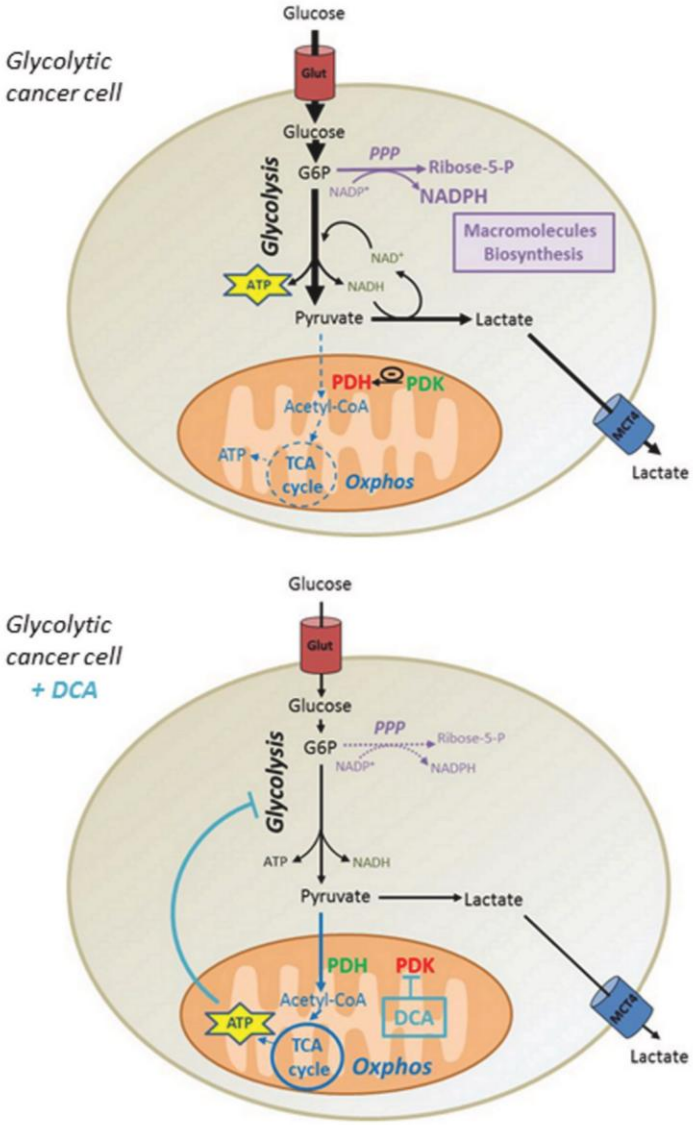

Figure 6: Mechanism by which DCA controls proliferation of glycolytic cancer cells. Highly glycolytic cancer cells exhibiting a Warburg phenotype ferment large amounts of glucose into lactate even in the presence of oxygen. This phenomenon allows rapid ATP production and provides precursors for biosynthetic processes promoting cell proliferation. By alleviating PDH inhibition by PDK, DCA fosters the conversion of pyruvate into acetyl-CoA and activates mitochondrial respiration. The consequent allosteric inhibition of glycolysis by ATP reduces glucose consumption and glycolytic intermediates levels, thereby decreasing the flux of the pentose phosphate pathway along with cellular proliferation. PDH, Pyruvate Dehydrogenase. PDK, Pyruvate Dehydrogenase Kinase. G6P, Glucose-6-phosphate. NAD, Nicotinamide Adenine Dinucleotide. NADP, Nicotinamide Adenine Dinucleotide Phosphate. Glut, Glucose Transporter. MCT4, Monocarboxylate Transporter 4.

maintain low ATP levels in order to avoid the allosteric inhibition of rate-limiting glycolytic enzymes (the Pasteur Effect) and to keep elevated glycolytic flux rates [10]. On the other hand, we showed that glycolysis promotes cancer cell proliferation, supporting the clinically tested strategies that inhibit the Warburg Effect [12, 25]. The positive relationship between glycolytic efficiency (moles of lactate produced per mole of glucose consumed) and the proliferation rate of cancer cells highlighted that rapidly dividing cells ferment large amounts of glucose in lactate. Recent studies have proposed that lactate production allows cancer cells to efficiently regenerate NAD^+ by the enzyme lactate dehydrogenase [26]. By maintaining NAD^+/NADH redox balance, cancer cells allow faster glucose flux through glycolysis, along with faster incorporation of glucose metabolites into biosynthetic pathways, thereby conferring advantages for proliferation [10].

To test whether enhanced aerobic glycolysis promotes cancer cells proliferation by fuelling anabolic processes, involvement of the pentose phosphate pathway (PPP) was evaluated. Indeed, it is known that the PPP uses glucose-6-phophate (the product of the glycolytic enzyme hexokinase) to supply cells with nucleotides and NADPH, a crucial reductant in anabolic reactions [20]. In our study, we showed that PPP flux, evaluated by the $NADPH_{ppp}$/$NADP^+$ ratio, was enhanced in Warburg-phenotype as compared to oxidative cancer cells. Comparison of the activity of PPP enzymes could also have been assayed to confirm this difference in PPP flux. Nevertheless, our results support that activation of glycolysis is accompanied by an increase in PPP activity for biosynthesis in rapidly dividing cells [20]. Convincingly, we observed that inhibition of the PPP decreased cancer cell proliferation, with a major effect in Warburg-phenotype cancer cells. The different sensitivity of glycolytic and oxidative cancer cells to PPP inhibition is likely due to the variable reliance on this pathway for macromolecules synthesis; such as lipids and nucleotides. In addition, cancer cells with elevated mitochondrial activity may compensate NADPH levels by using TCA cycle-associated enzymes (malic enzymes and isocitrate dehydrogenases) to replenish the NADPH pool [20]. Taken together, these results demonstrate that cancer cells, especially aggressive Warburg-phenotype cancer cells, rely on the pentose phosphate pathway for optimal proliferation.

To evaluate the therapeutic relevance of these findings, the effects of DCA were investigated. DCA has already shown interesting anticancer properties *in vitro* and *in vivo* [17], and is currently tested in Phase I-II clinical trials [12]. To date, DCA owes its therapeutic properties to the re-sensitization of cancer cells to apoptosis [17]. However, as recently pointed out by Stockwin et al. [27], DCA is relatively inactive on cell viability and induces apoptosis only at high concentrations. Stockwin et al. further reported that DCA was preferentially active in cells with mitochondrial defect. Therefore, we postulated that, at lower dose, DCA also decreases cancer cells proliferation by reducing glycolysis. We found that 5 mM DCA was more effective in Warburg-phenotype cancer cells, reducing cell proliferation by decreasing glycolysis. The different sensitivities among cell lines with different metabolic phenotype may result from the capacity of DCA to reach PDK in the matrix of mitochondria. Indeed, like pyruvate, DCA is ionized and cannot pass through membranes. Curiously, there have been only few reports on the transport of DCA in the cytosol of mammalian cells [28, 29]. Although not yet investigated, the level of expression of mitochondrial pyruvate transporters (MPCs) may also define DCA efficacy. Furthermore, as DCA has a different *Ki* for each of the four PDK isoenzymes [30], the variable sensitivity of cancer cells may result from de differential expression of PDKs.

Although some reports showed that DCA in comparable concentrations promotes cytotoxicity in other cell lines [31, 32], we showed that DCA had no incidence on MDA-MB-231 and SiHa cancer cells viability. Based on our data, we propose that the reactivation of ATP production in mitochondria induced by DCA lowers the glycolytic flux of cancer cells, thereby reducing the incorporation of glucose. Consequently, the decrease in the synthesis of glycolytic intermediates reduces the activity of biosynthetic pathways such as the PPP, compromising the proliferation of Warburg-phenotype cancer cells (Figure 6). Nevertheless, it is conceivable that other mechanisms may participate to the anti-proliferative effects of DCA, as other studies have shown cell cycle arrest at comparable DCA concentrations [33, 34].

Overall, our study showed that the PPP bridges glycolysis and proliferation in aggressive cancer cells, and pleads for considering cancer metabolism for the choice of adequate therapeutic anticancer strategies. In particular, joining the conclusions of others [27], we suggest that clinical development of DCA may benefit from selecting patients with highly glycolytic tumors.

MATERIALS AND METHODS

Cell culture and reagents

SiHa human cervix squamous cell carcinoma (ATCC), MDA-MB-231 human breast cancer (ATCC), TLT (transplantable liver tumor) mouse hepatocarcinoma [35], FSaII mouse fibrosarcoma [36], KHT mouse sarcoma [37], NT2 mouse mammary tumor [38] were grown following provider's recommendation or as described. SiHa with partial mitochondrial depletion (ρ0) were obtained by chronic exposure to low concentrations of ethidium bromide as detailed previously [14, 39]. All cultures were kept at 37°C in 5% CO_2 atmosphere. Cells

were incubated in a unique experimental medium 24 hours before the experiments (DMEM without glutamine [Invitrogen], containing 4.5 g/L glucose supplemented with 10 % heat inactivated FBS and 1% penicillin-streptomycin). When SiHa ρ0 were compared to SiHa wild-type, DMEM without glutamine (Invitrogen), containing 4.5 g/L glucose supplemented with 1% pyruvate, 10 % heat-inactivated FBS, 1% penicillin-streptomycin and 50 ng/ml uridine was used. Unless stated otherwise, experiments were performed on confluent cells. Oligomycin in an ATP synthase inhibitor. Dichloroacetate (DCA) is a pyruvate dehydrogenase kinase (PDK) inhibitor. 6-aminonicotinamide (6-AN) is an inhibitor of $NADP^+$-dependent enzymes of the pentose phosphate pathway, glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6-PGD). All chemicals were purchased from Sigma. Oligomycin was dissolved in DMSO. DCA and 6-AN were directly dissolved in culture media.

siRNA transfection

ON-TARGETplus SMARTpool siRNA against human G6PD and ON-TARGETplus Non-targeting siRNA were from Dharmacon. Final siRNA concentration was 25 nM. All siRNA were transfected using RNAi/MAX according to manufacturer's instructions (Invitrogen).

Western blotting

Whole cell lysates were collected and subjected to immunoblot analysis as previously described [40]. Primary antibodies were human monoclonals against G6PD or Hsp90 (Sigma).

Mitochondrial oxygen consumption

The oxygen consumption rate (OCR) of intact whole cells was measured using a Bruker EMX EPR spectrometer operating at 9.5 GHz as previously described [41]. Adherent cells were harvested in fresh experimental medium (10^7 cells/ml). 100 µl of the cell suspension were mixed with 100 µl of 20% dextran to avoid agglomeration and cells were sealed in a glass capillary tube in the presence of 0.2 mM of a nitroxide probe acting as an oxygen sensor (^{15}N 4-oxo-2,2,6,6-tetramethylpiperidine-d_{16}-^{15}N-1-oxyl, CDN isotopes, Pointe-Claire, Quebec, Canada). Cells were maintained at 37°C during the acquisition of the spectra. EPR linewidth was measured every minute and reported on a calibration curve to obtain the oxygen concentration. OCR was determined by the absolute value of the slope of the decrease in oxygen concentration in the closed capillary tube over time [42]. To obtain OCR from oxidative phosphorylation, OCR in the presence of 1 µM oligomycin treatment was subtracted from the total OCR of the cells.

Glucose consumption and lactate production

Glucose consumption and lactate production were measured from supernatants of the cultured cells. Metabolite concentrations were quantified on deproteinized samples using specific enzymatic assays on a CMA600 analyzer (CMA Microdialysis AB, Solna, Sweden). Glucose consumption and lactate production were normalized to protein content using the Pierce BCA Protein assay (Thermo Scientific). To efficiently detect differences in metabolite concentrations between SiHa WT and SiHa ρ0 supernatants, a low glucose (1 g/L) medium was used.

Intracellular ATP quantification

Total intracellular ATP was measured by the ATP Determination Kit (Invitrogen) according to manufacturer's protocol. Cells were washed twice with PBS and lysed in the buffer recommended by the manufacturer (10 mM Tris, 1 mM EDTA, 100 mM NaCl, 0.01% Triton X-100). Cell lysates were added to a reaction mixture containing luciferase and luciferine for bioluminescence measurements using a plate reader (SpectraMax M2e, Molecular Devices). A standard curve was generated with known ATP concentrations in the same conditions. A fraction of the cell lysates was systematically used for protein quantification (Pierce BCA Protein assay, Thermo Scientific) to normalize ATP level to the protein content.

Proliferation

Cell proliferation was assayed with a 5-bromo-2'-deoxyuridine (BrdU)-ELISA based kit (Roche) following provider's instructions. Sub-confluent cells were incubated in the presence of BrdU (a nucleotide analog) during 4 hours. When proliferation of SiHa WT and SiHa ρ0 were compared, cells were incubated during 6 hours in the presence of BrdU. The amount of BrdU incorporated in the cells was assessed by colorimetric measurements using a plate reader (SpectraMax M2e, Molecular Devices), which allowed the quantitation of DNA synthesis in replicating cells.

NADPH and $NADP^+$ measurements

$NADP^+$ and NADPH levels were detected individually from 6,000 harvested cells using a detection kit (NADP/NADPH-Glo Assay, Promega) according to manufacturer's instructions.

Statistics

All results are expressed as means ± standard error of the mean (SEM). Statistical analyses were performed using the GraphPad Prism 5 software. $P<0.05$ was considered statistically significant.

ACKNOWLEDGMENTS

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CONFLICTS OF INTERESTS

The authors have declared that no conflict of interest exists

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Inhibition of the pentose phosphate pathway by dichloroacetate unravels a missing link between aerobic glycolysis and cancer cell proliferation

Supplementary Material

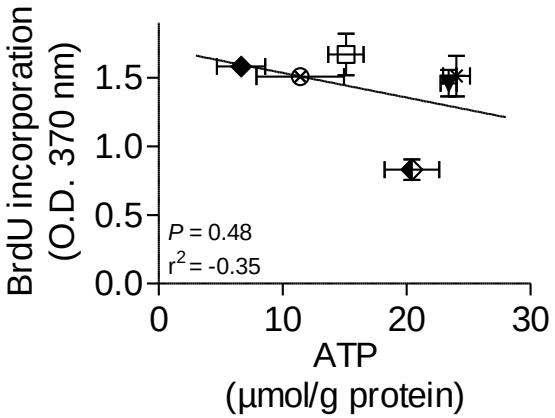

Fig. S1. DNA synthesis is not correlated to intracellular ATP content in cancer cells. Measurements were performed after 24 h incubation in the presence of a culture medium containing only glucose as energetic fuel. Total ATP was quantified from lysed cells and normalized to protein content. Proliferation rates were analyzed by the incorporation of a nucleotid analog (5-bromo-2'-deoxyuridine [BrdU]). A non-significant correlation was found (*p*-value = 0.48, Pearson r = -0.35). Results are expressed as means ± SEM.

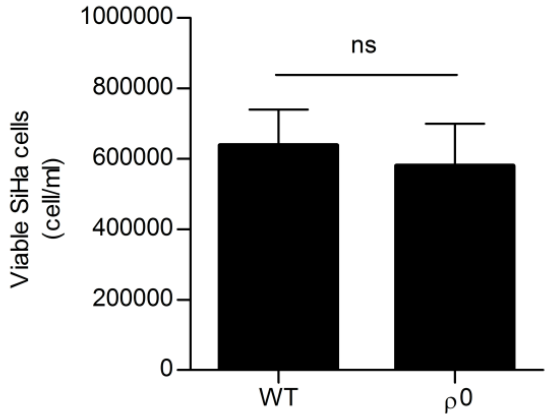

Fig. S2: Viability assays show no difference in the number of viable WT and ρ0 SiHa cancer cells. Viability assays were performed on wild-type (WT) and mitochondria-deficient (ρ0) SiHa cancer cells using trypan blue exclusion after 24 h incubation in the experimental medium (DMEM without glutamine, containing 4.5 g/L glucose supplemented with 10 % heat inactivated FBS and 1% penicillin-streptomycin. Results are expressed as means ± SEM. Two-sided *t* test. ns, non-significant.

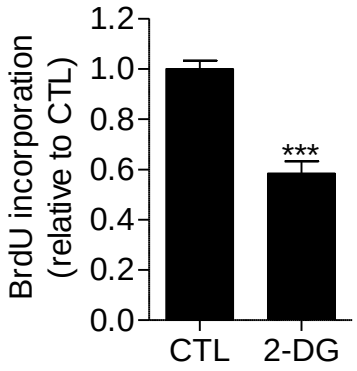

Fig. S3: Treatment with the glycolysis inhibitor 2-Deoxy-D-glucose impairs proliferation of MDA-MB-231 cancer cells. MDA-MB-231 cancer cells were exposed to 5 mM 2-Deoxy-D-glucose (Sigma) during 48 h. Proliferation rates were analyzed by the incorporation of a nucleotid analog (5-bromo-2'-deoxyuridine [BrdU]) incubated during 4 h in the presence of the cells. Two-sided *t* test. ****p*<0.001. Results are expressed as means ± SEM.

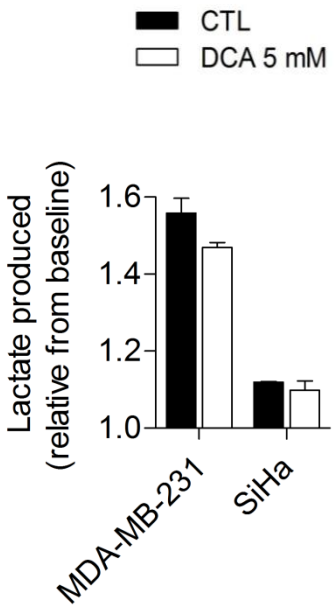

Fig. S4: Short-term lactate production measurements reveal that DCA is more effective in Warburg-phenotype cancer cells. Lactate production by MDA-MB-231 and SiHa cancer cells treated or non-treated with DCA 5 mM during 1 h. Results are expressed as the relative change in lactate concentration from baseline.

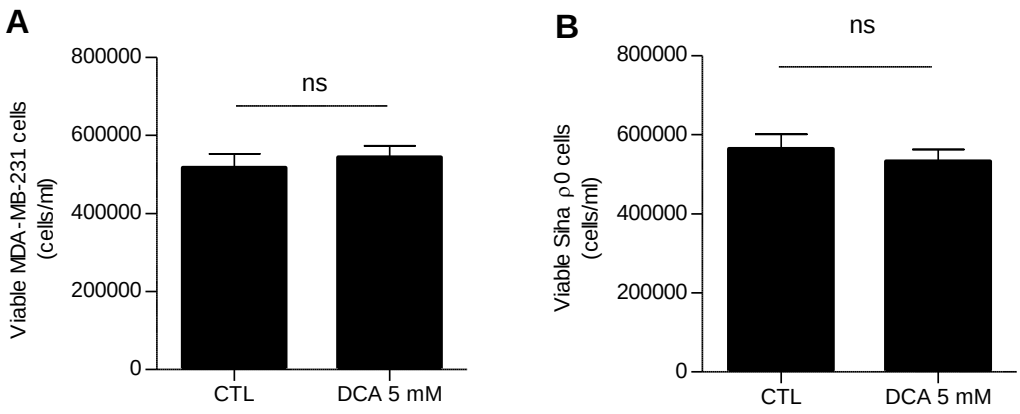

Fig. S5: DCA treatment does not induce cancer cell death. Viability assays using trypan blue exclusion were performed on MDA-MB-231 cancer cells (A) and SiHa ρ0 cancer cells (B) treated or non-treated with DCA 5 mM during 48 h. The absence of significant difference in viable cell number between treated and non-treated cells indicated that cell mortality was not induced by DCA treatment. Results are expressed as means ± SEM. Two-sided *t* test. ns, non-significant.

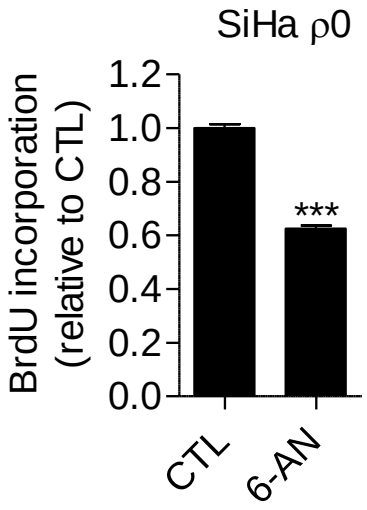

Fig. S6: Treatment with 6-AN impairs proliferation of SiHa ρ0 cancer cells. SiHa ρ0 cancer cells were exposed to 100 µM 6-AN during 48 h. Proliferation rates were analyzed by the incorporation of a nucleotid analog (5-bromo-2'-deoxyuridine [BrdU]) incubated during 4 h in the presence of the cells. Two-sided *t* test. ****p*<0.001. Results are expressed as means ± SEM.

Chapter 4 Hydrogen sulfide donor potentiates radiotherapy

A fast hydrogen sulfide-releasing donor increases the tumor response to radiotherapy

Géraldine De Preter, Caroline Deriemaeker, Pierre Danhier, Lucie Brisson, Thanh Trang Cao Pham, Vincent Grégoire, Bénédicte F. Jordan, Pierre Sonveaux and Bernard Gallez.

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Cancer Biology and Signal Transduction

Molecular Cancer Therapeutics

A Fast Hydrogen Sulfide–Releasing Donor Increases the Tumor Response to Radiotherapy

Géraldine De Preter[1], Caroline Deriemaeker[1], Pierre Danhier[1], Lucie Brisson[2], Thanh Trang Cao Pham[1], Vincent Grégoire[3], Bénédicte F. Jordan[1], Pierre Sonveaux[2], and Bernard Gallez[1]

Abstract

Hydrogen sulfide (H_2S) is the last gaseous transmitter identified in mammals, and previous studies have reported disparate conclusions regarding the implication of H_2S in cancer progression. In the present study, we hypothesized that sodium hydrosulfide (NaHS), a fast H_2S-releasing donor, might interfere with the mitochondrial respiratory chain of tumor cells, increase tumor oxygenation, and potentiate the response to irradiation. Using electron paramagnetic resonance (EPR) oximetry, we found a rapid increase in tumor pO_2 after NaHS administration (0.1 mmol/kg) in two human tumor models (breast MDA-MB-231 and cervix SiHa), an effect that was due to a decreased oxygen consumption and an increased tumor perfusion. Tumors irradiated 15 minutes after a single NaHS administration were more sensitive to irradiation compared with those that received irradiation alone (increase in growth delay by 50%). This radiosensitization was due to the oxygen effect, as the increased growth delay was abolished when temporarily clamped tumors were irradiated. In contrast, daily NaHS injection (0.1 mmol/kg/day for 14 days) did not provide any effect on tumor growth *in vivo*. To understand these paradoxical data, we analyzed the impact of external factors on the cellular response to NaHS. We found that extracellular pH had a dramatic effect on the cell response to NaHS, as the proliferation rate (measured *in vitro* by BrdU incorporation) was increased at pH = 7.4, but decreased at pH = 6.5. Overall, our study highlights the complex role of environmental components in the response of cancer cells to H_2S and suggests a new approach for the use of H_2S donors in combination with radiotherapy. *Mol Cancer Ther; 15(1); 154–61. ©2015 AACR.*

Introduction

Hydrogen sulfide (H_2S) has emerged as an important endogenous modulator and is now considered the third member of the gasotransmitter family, along with nitric oxide (NO) and carbon monoxide (CO; ref. 1). H_2S is an enzymatically produced small molecule that can freely cross biologic membranes and exert a wide range of actions. H_2S most known effect is the reversible inhibition of the last mitochondrial electron acceptor, cytochrome C oxidase (2). Other H_2S targets have been reported so that H_2S appears also implicated in the cardiovascular system. H_2S dilates rat and human blood vessels by opening smooth muscle cells K_{atp} channels (3). Other cardiovascular effects of H_2S, such as protection against ischemia/reperfusion injury, have been described (4). Moreover, H_2S effects are also found in the nervous and endocrine systems, as well as in inflammation (5). Since the discovery that H_2S is important for physiologic and pathologic processes, development and clinical applications of injectable donors allowing controlled and safe administration of the molecule have attracted growing interest (6). Because of their commercial availability, preclinical and clinical studies have been conducted using fast H_2S-releasing inorganic salts, such as sodium hydrosulfide (NaHS) and sodium sulfide (Na_2S; ref. 7).

Until now, investigations in cancer research have mainly focused on the effects of H_2S on proliferation and survival of cancer cells (8–11). However, controversial results exist, as evidenced by the reported increased (8) or decreased (9) proliferation of colon cancer cells following NaHS treatment. A study showed that H_2S derived from NaHS may confer intrinsic radioresistant properties to cancer cells (12). However, the beneficial effects of H_2S as cotreatment to radiotherapy have never been studied *in vivo*. We paid attention to this latter aspect because it was previously shown that NO, another gaseous mediator, radiosensitizes tumors in mice (13–15). It was shown that NO acts as an intrinsic radiosensitizer but also acts through an "oxygen enhancement" effect, alleviating tumor hypoxia, which is a major cause of resistance to radiotherapy (16, 17). pO_2 values of 2.5 mmHg or less are characteristic of advanced solid tumors in a wide range of human cancers (18), and oxygen tensions below 10 mmHg are estimated to significantly reduce radiosensitivity (19). It is related to the fact that oxygen enhances water radiolysis and fixes DNA damage following radiation treatment.

[1]Biomedical Magnetic Resonance Research Group, Louvain Drug Research Institute (LDRI), Université catholique de Louvain, Brussels, Belgium. [2]Pole of Pharmacology and Therapeutics, Institute of Experimental and Clinical Research (IREC), Université catholique de Louvain, Brussels, Belgium. [3]Pole of Molecular Imaging, Radiotherapy and Oncology, Institute of Experimental and Clinical Research (IREC), Université catholique de Louvain, Brussels, Belgium.

Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Corresponding Author: Bernard Gallez, Université catholique de Louvain, LDRI-REMA, Avenue Mounier 73.08, B-1200 Brussels, Belgium. Phone: 0032-2-7647391; Fax: 0032-2-7647390; E-mail: bernard.gallez@uclouvain.be

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In the present study, we considered the potential effect of NaHS administration on tumor hypoxia and response to irradiation. Considering the origins of tumor hypoxia (18), the effects of NaHS treatment on the delivery of oxygen from the blood and on oxygen consumption by cancer cells were examined. We also investigated *in vitro* and *in vivo* impact of NaHS on cancer cell growth when used as a single therapeutic compound.

Materials and Methods

Cell culture and reagents

The human cervix carcinoma SiHa and the human breast cancer MDA-MB-231 cell lines were from the American Type Culture Collection (ATCC). SiHa cancer cells were obtained in 2012, and MDA-MB-231 cancer cells were obtained in 2011. Cell lines were authenticated by the provider and were frozen in liquid nitrogen soon after arrival. In this study, aliquots were thawed and early passage (<20 passages) cells were used. Cells were grown in DMEM + Glutamax (Life Technologies) containing 4.5 g/L glucose supplemented with 10% heat inactivated FBS and 1% penicillin–streptomycin. For the experiments, culture medium containing no glutamine was used. pH was buffered with 10 mmol/L PIPES or 3.7 g/L sodium bicarbonate. For the oxygen consumption measurements, where no incubation time was used, pH was adjusted to 6.5, 7.0, or 7.5 with HCl 0.1 mol/L and NaOH 0.1 mol/L solutions. All cultures were kept at 37°C in 5% CO_2 atmosphere. NaHS (Sigma) crystals were dissolved in physiologic saline (NaCl 0.9%). Rotenone (Sigma), a mitochondrial complex I inhibitor, was diluted in DMSO. Solutions were freshly prepared before all experiments.

Oxygen consumption rate

The oxygen consumption rate (OCR) of intact whole cells was measured using a Bruker EMX EPR spectrometer operating at 9.5 GHz as previously described (20). Adherent cells were trypsinized and resuspended in fresh medium (10^7 cells/mL). Hundred μL of the cell suspension was mixed with 100 μL of 20% dextran to avoid agglomeration and was sealed in a glass capillary tube in the presence of 0.2 mmol/L of a nitroxide probe acting as an oxygen sensor (^{15}N 4-oxo-2,2,6,6-tetramethylpiperidine-d_{16}-^{15}N-1-oxyl, CDN isotopes). Cells were maintained in 37°C during the acquisition of the spectra. EPR linewidth was measured every minute and reported on a calibration curve to obtain the oxygen concentration (13). OCR was determined by the absolute value of the slope of the decrease in oxygen concentration in the closed capillary tube.

Cell proliferation

Cell proliferation was assayed with a 5-bromo-2′-deoxyuridine (BrdU)–ELISA-based method (Roche) following the provider's instructions. Cells were incubated in the presence of BrdU (a nucleotide analogue) during 4 hours, and the amount of BrdU incorporated in the cells was assessed by colorimetric measurements using a plate reader (SpectraMax M2e; Molecular Devices).

Glucose consumption

Extracellular glucose consumption was measured from supernatant of cultured cells. Metabolite concentration was enzymatically quantified on deproteinized samples with a CMA600 analyzer (CMA Microdialysis AB). Glucose consumption was normalized to protein content using the Pierce BCA Protein assay (Thermo Scientific).

Intracellular ATP quantification

Total intracellular ATP was measured by the ATP Determination Kit (Life Technologies) according to the manufacturer's protocol. Cells were washed twice with PBS and lysed in the buffer recommended by the manufacturer (10 mmol/L Tris, 1 mmol/L EDTA, 100 mmol/L NaCl, 0.01% Triton X-100). Cell lysates were added to a reaction mixture containing luciferase and luciferine for bioluminescence measurements using a plate reader (SpectraMax M2e; Molecular Devices). A standard curve was generated with known ATP concentrations in the same conditions. Intracellular ATP concentration was normalized to protein content using the Pierce BCA Protein assay (Thermo Scientific).

pHi measurements

Cells were incubated for 30 minutes at 37°C in Hank's medium (Sigma) containing 7 μmol/L 5-(and-6)-Carboxy SNARF-1, Acetoxymethyl Ester, a fluorescent pH indicator (Life Technologies). Cells were washed with Hank's medium, and fluorescence (excitation 485 nm; emission 580 and 642 nm) was detected using a plate reader (SpectraMax i3; Molecular Devices). Fluorescent values were converted into pH values using the nigericin/high K^+ solution calibration technique according to the manufacturer.

Mouse models and *in vivo* experiments

Five-week-old female NMRI nude mice (Janvier Labs) were intramuscularly injected with 10^7 SiHa or MDA-MB-231 human cancer cells in the rear leg. Tumor xenografts were allowed to grow up to 8 mm before experimentation. For the treated groups, NaHS was dissolved in physiologic saline (NaCl 0.9%) and given by intraperitoneal injection (100 μmol/kg body weight). Control animals were treated with physiologic saline only. Animals were anesthetized by inhalation of isoflurane mixed with air (3% induction, 1.8% maintain for a minimum of 15 minutes before any measurement). All animal experiments were conducted in accordance with national animal care regulations.

Tumor oxygenation

EPR oximetry using charcoal (CX 0670-1; EM Sciences) as oxygen sensor was used to dynamically evaluate changes in tumor oxygenation after treatment with NaHS, using a protocol described previously (21). EPR spectra were recorded using an EPR spectrometer (Magnettech), with a low-frequency microwave bridge operating at 1.2 GHz and an extended loop resonator. A suspension of charcoal was injected into the center of the tumor 1 day before measurement (100 mg/mL; 50 μL injected, particle size of 1–25 μm). The localized EPR measurements correspond to an average of the pO_2 values in a volume of approximately 10 mm^3 (22). For the experiments, baseline values were performed after mice were anesthetized to determine the oxygen status of tumors before injection of the treatment. Then, the effect of NaHS was measured by following tumor pO_2 for 1 hour after the single injection. Body temperature of the mice was kept at 37°C throughout the experiment.

Tumor perfusion

The Patent blue staining method was used to obtain an estimation of the tumor perfusion fraction using a protocol described previously (23). Fifteen minutes after NaHS or physiologic saline treatment, 100 µL of Patent blue (Sigma) solution (1.25%) was injected in the tail vein of the mice. After 1 minute, mice were sacrificed and tumors were excised. To evaluate the tumor perfusion fraction, each tumor was cut into two size-matched halves, and the percentage of stained area of the whole cross-section was determined using an in-house program running on MatLab.

Tumor radioresponse

The tumor was locally irradiated (^{137}Cs γ-irradiator) with a single dose of 16 Gy. Mice were anesthetized, and the tumor was centered in a 3-cm diameter circular irradiation field. Irradiation was given 15 minutes after injection of NaHS or physiologic saline. After radiotherapy, tumor growth was determined using a caliper until the diameter reached 14 mm, time at which the mice were sacrificed.

Statistical analysis

All results are expressed as mean ± SEM. Differences between groups were analyzed using the unpaired Student *t* test or ANOVA when more than two groups were compared. The value of $P < 0.05$ was considered statistically significant.

Results

NaHS injection increases oxygenation of hypoxic tumors in mice

Hypoxia is a major cause of resistance to radiotherapy in solid tumors. Therefore, we analyzed the capability of the fast H_2S-releasing donor NaHS to increase tumor oxygenation in two human tumor models. As H_2S is rapidly oxidized in biologic samples, local tumor oxygenation was monitored before (baseline) and during 1 hour after intraperitoneal injection of NaHS (100 µmol/kg) or vehicle [physiologic saline (NaCl 0.9%)]. Oxygen levels were quantified using EPR oximetry, a sensitive method allowing continuous measurement of pO_2 from the same site over time (21). Our results showed that, as compared with control groups, NaHS injection rapidly increased tumor pO_2 in human breast MDA-MB-231 (Fig. 1A) and human cervix SiHa (Fig. 1B) xenografts, where significant increased oxygenation was observed 15 minutes after NaHS injection. Note the different scales used in Fig. 1A and Fig. 1B. Interestingly, the significant effect on tumor pO_2 correlated with the time to reach maximum plasmatic H_2S concentration following an intraperitoneal injection of NaHS (24).

NaHS inhibits cellular oxygen consumption and enhances tumor perfusion

To understand the significant increase in pO_2 induced by NaHS in hypoxic tumors, oxygen consumption of cancer cells was first studied. We intended to determine *in vitro* how NaHS influences the OCR of MDA-MB-231 and SiHa cancer cells. In aqueous solution, H_2S derived from dissolved NaHS is in equilibrium with the poorly membrane permeant $HS^- + H^+$ with a pKa of 6.9 (25). We therefore analyzed the influence of different extracellular pH (pHe) on OCR inhibition by NaHS.

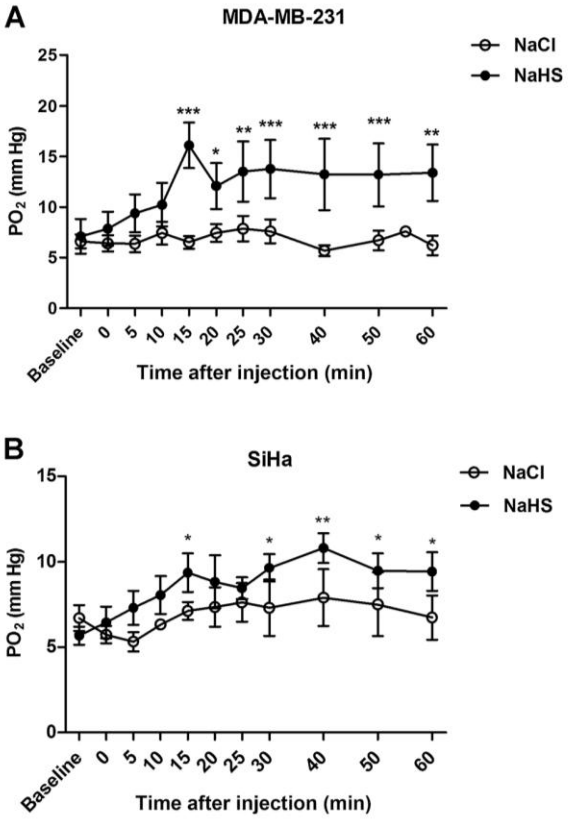

Figure 1.
NaHS injection increases tumor pO_2. Tumor pO_2 was monitored in MDA-MB-231 (A) and SiHa (B) tumors by EPR (L-band) oximetry before (baseline) and after NaHS (100 µmol/kg, ●) or vehicle (NaCl 0.9%, ○) i.p. injection (60 minutes). Each point represents mean pO_2 ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. ANOVA and Bonferroni post-test ($n = 5$–7/group).

As shown in Fig. 2A and B, exposure to 50 µmol/L NaHS instantaneously inhibited OCR in MDA-MB-231 and SiHa tumor cell lines, and the response was dependent on pHe. We observed that OCR inhibition was more effective when pHe decreased. After that to examine the concentration response to NaHS, MDA-MB-231 and SiHa cells were treated with 0, 1.5, 25, 50, and 100 µmol/L NaHS in the presence of acidic pHe (Fig. 2C and D). We observed a significantly reduced OCR at 50 µmol/L NaHS in both cancer cell lines. At lower sulfide concentration, the affinity of H_2S for the heme center of cytochrome C oxidase is too low to produce detectable inhibition of the enzyme (2), justifying the absence of OCR inhibition at lower NaHS concentrations. Hence, the trend toward an increased OCR in cells exposed to 1.5 µmol/L NaHS corroborates that H_2S may also act as a mitochondrial electron donor when present in low concentration (26). At 100 µmol/L NaHS, the same OCR inhibition as with rotenone, an inhibitor of mitochondrial respiration, was observed. To ensure that OCR inhibition was not due to cell mortality caused by the experimental conditions, viability assays were also performed. As shown in Supplementary Fig. S1A–S1D, no cell death was found.

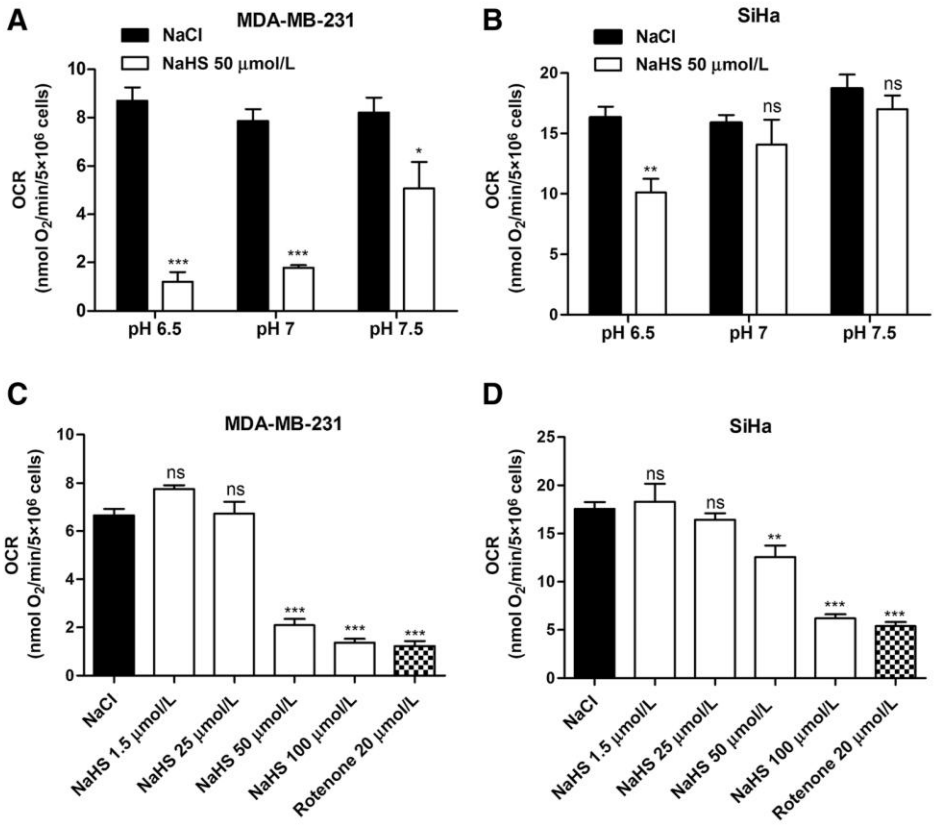

Figure 2.
NaHS treatment decreases cancer cells OCR. OCR of viable MDA-MB-231 and SiHa cancer cells was measured *in vitro* using EPR (X-band) oximetry. A and B, cancer cells treated with 50 μmol/L NaHS or vehicle (NaCl 0.9%) in the presence of different pHe values. C and D, cancer cells treated with increasing NaHS concentration or vehicle in the presence of pHe = 6.5. Each bar represents mean OCR ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant. Two-sided *t* test (A and B; $n = 3$) or ANOVA and Dunnett post-test (C and D; $n = 3$).

Blood perfusion was also investigated. Measurements were performed 15 minutes after NaHS injection using the Patent blue staining assay. This method, involving the injection of a dye in the systemic circulation of the mice, has previously been validated and compared with DCE-MRI (23). We observed that, as compared with vehicle-treated mice, the perfused area was increased in MDA-MB-231 (Fig. 3A) and SiHa (Fig. 3B) xenografts of NaHS-treated (100 μmol/kg) mice, indicating that increased perfusion also accounts for the improved tumor O_2 level following NaHS treatment.

NaHS radiosensitizes tumors by an "oxygen enhancement" effect

To investigate the therapeutic relevance of NaHS as a potential radiosensitizer, regrowth delay assays were performed in MDA-MB-231 tumors. Tumor growth curves are presented in Fig. 4A. Without irradiation, no difference in tumor growth was observed after a single i.p. administration of NaHS or vehicle. In irradiated groups, the regrowth delay to reach a 12-mm tumor diameter was 13.6 ± 2 days for irradiation + vehicle and 20.5 ± 3.5 for irradiation + NaHS, suggesting that NaHS administration 15 minutes before irradiation increased sensitivity of tumors by a factor of 1.5 (Fig. 4B). To highlight that NaHS radiosensitizes tumor through an oxygen effect, we also used a group of mice receiving irradiation + NaHS whose legs were temporarily ligated to induce complete hypoxia at the time of irradiation. As the regrowth delay was similar to the irradiation + vehicle group

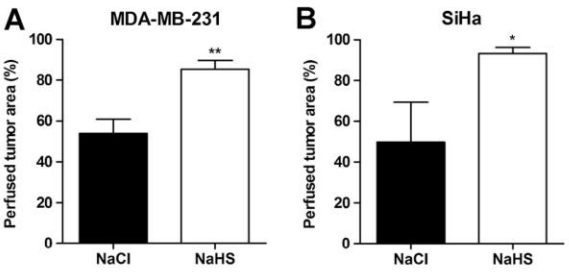

Figure 3.
NaHS injection increases blood perfusion. Perfusion of MDA-MB-231 (A) and SiHa (B) tumors was measured by Patent blue staining 15 minutes after NaHS (100 μmol/kg) or vehicle (NaCl 0.9%) i.p. injection. Each bar represents mean colored area ± SEM. *, $P < 0.05$; **, $P < 0.01$. Two-sided *t* test ($n = 4$–5/group).

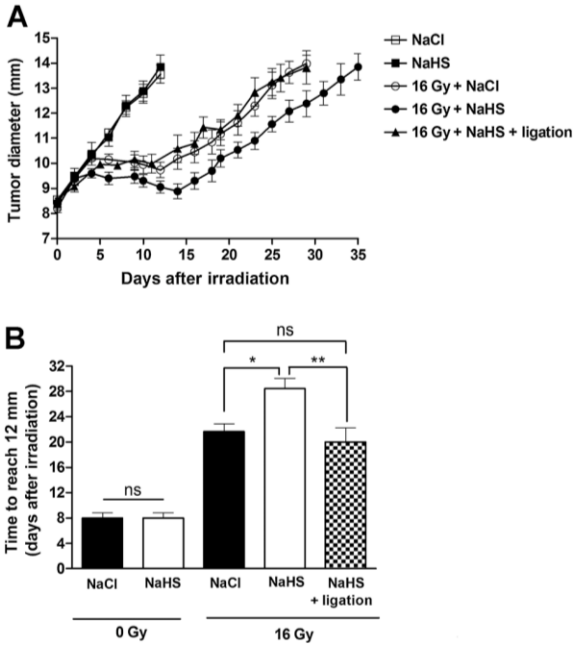

Figure 4.
NaHS in combination with radiotherapy increases the radioresponse of MDA-MB-231 tumors. A, tumor growth curves of mice treated with NaHS (100 μmol/kg, ■) or vehicle (NaCl 0.9%, □) alone, 16 Gy of radiotherapy 15 minutes after NaHS (●) or vehicle (○) injection, and 16 Gy of radiotherapy 15 minutes after NaHS injection plus ligation at the time of irradiation (▲). Each point represents the mean tumor size ± SEM. B, regrowth delay expressed as the time to reach a tumor size of 12 mm. *, $P < 0.05$; **, $P < 0.01$; ns, not significant. ANOVA and Bonferroni post-test ($n = 6$/group).

(Fig. 4B), this experiment showed that oxygen was necessary for NaHS to increase radioresponse.

Chronic NaHS injection alone is inactive to control tumor growth in mice

The potential inhibitory or stimulatory effect of the H_2S donor on tumor growth was also evaluated *in vivo*. Nude mice bearing MDA-MB-231 xenografts were daily i.p. injected with NaHS (100 μmol/kg/day) or vehicle (physiologic saline), and tumor diameter was measured until tumors reached 14 mm in diameter. Results showed that daily NaHS injection was inactive to control tumor growth, as no difference between NaHS-treated and vehicle-treated MDA-MB-231 tumor-bearing mice was found (Fig. 5A). Chronic NaHS injections seemed to have low incidence on the general condition of the mice, as no deterioration in body weight was observed as compared with vehicle-treated mice during the experiment (Fig. 5B).

Prolonged exposure to NaHS exhibits opposite effects on cancer cell proliferation depending on the extracellular pH

Some reports have already investigated the intrinsic anticancer properties of H_2S. Under different circumstances, H_2S acts as an inhibitor or as a promoter of proliferation for various cell types (27). It is currently hypothesized that conflictual conclusions arise from the manner in which cells are exposed to the treatment. We investigated whether pHe could play a role in the effects of NaHS on cancer cell proliferation. For the purpose, MDA-MB-231 cancer cells were incubated with 50 μmol/L NaHS in alkaline (pH 7.4) or acidic (pH 6.5) media during 4 hours. Proliferation was assessed using quantitation of BrdU incorporated in the DNA of the cells during incubation. Our results showed opposite effects of NaHS depending on pHe (Fig. 6A). In the presence of an alkaline pHe, DNA synthesis in NaHS-treated cells increased, as compared with nontreated cells. In contrast, a decrease in DNA synthesis was induced by the H_2S donor when cells were incubated in an acidic medium. The decreased proliferation found in cells incubated at low pHe was not associated with cell mortality (Supplementary Fig. S2). Further experiments were conducted to help understand the complex role of H_2S in cancer cell proliferation. We found that reactive oxygen species were not implicated in the pro- or antiproliferative effects of NaHS (Supplementary Fig. S3). We then asked whether a glycolytic switch occurred in the NaHS-treated cells. Indeed, enhanced glycolysis is known to confer advantages for cancer cell proliferation by providing reductive equivalents and glycolytic intermediates that fuel important biosynthetic reactions and promote cell expansion (28–30). We found a significant increase in glucose consumption (Fig. 6B) in NaHS-treated cells compared with nontreated cells in the alkaline condition, seemingly induced to maintain ATP homeostasis (Fig. 6C) in compensation to inhibition of the mitochondrial function. Supporting our findings, others have

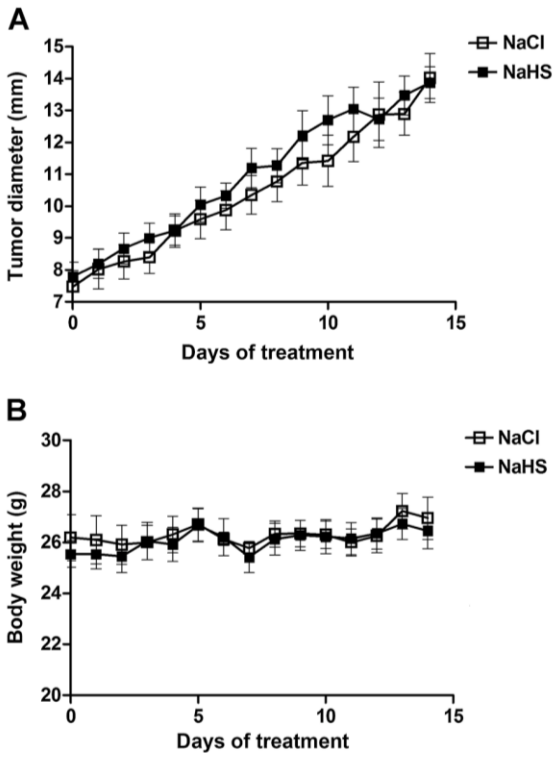

Figure 5.
NaHS injected chronically is inactive on MDA-MB-231 tumor growth. Tumor growth (A) and body weight (B) of mice treated daily with NaHS (100 μmol/kg/day, ■) or vehicle (NaCl 0.9%, □). Each point represents mean ± SEM ($n = 4$/group).

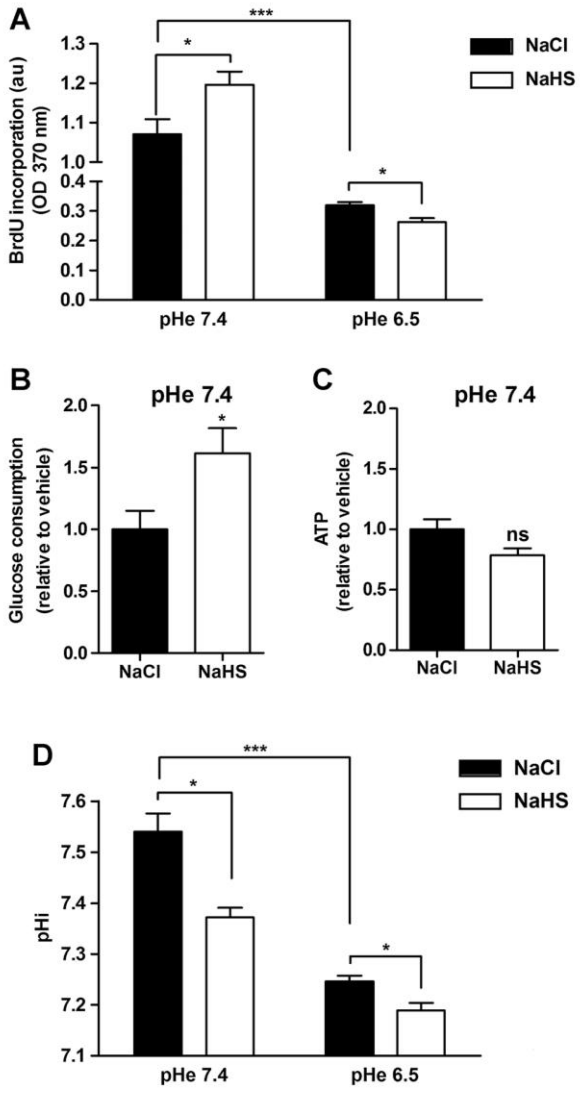

Figure 6.
pHe-dependent opposite effects of NaHS on MDA-MB-231 cancer cell proliferation. Cancer cells were incubated with 50 µmol/L NaHS or vehicle (NaCl 0.9%) in the presence of different pHe values during 4 hours. A, proliferation rates were analyzed by incorporation of a nucleotide analogue (BrdU) in the DNA of the cells during the incubation. B, glucose consumption was evaluated by measuring extracellular glucose concentrations before and after the 4-hour incubation in the presence of pHe = 7.4. C, intracellular ATP level was quantified using a luciferase-based method after the 4-hour incubation in the presence of pHe = 7.4. D, pHi was measured using a fluorescent pH indicator. Each bar represents mean ± SEM. *, $P < 0.05$; ***, $P < 0.001$; ns, not significant. Two-sided t test ($n \geq 3$).

also reported that a synthetic H_2S donor promotes an influx of glucose and triggers enhanced glycolysis in another human breast cancer cell line (31). We then analyzed the decreased proliferation induced by NaHS in the acidic condition. By noticing that low pHe itself had profound impact on cancer cell proliferation, we questioned whether NaHS treatment was able to exacerbate the acidic stress experienced by cancer cells at low pHe. Indeed, it was recently evidenced that, besides its ability to enhance glycolysis, H_2S also impairs the activity of pH regulators in cancer cells, leading to the intracellular accumulation of acid and reduction of pHi (31, 32). It is also known that pHi must be kept in a narrow range, otherwise cell-cycle progression and biosynthetic processes are compromised (33). By measuring pHi in MDA-MB-231 cancer cells, we observed that 4 hours of exposure to an acidic pHe decreased pHi and that an additional effect was found when NaHS was added (Fig. 6D).

Discussion

In this study, we showed that H_2S used as cotreatment to radiotherapy, but not as single treatment, provided beneficial effects for cancer therapy.

Clinical investigation has demonstrated that tumor hypoxia, arising from an imbalance between oxygen consumption and blood supply, is a major cause of resistance to radiotherapy. Therefore, selective targeting of cellular oxidative metabolism and/or tumor perfusion is challenging to radiosensitize tumors. For the first time, we report that administration of an H_2S donor (NaHS) before radiotherapy improves tumor oxygenation and radiosensitivity. Our results suggest that NaHS rapidly reduces tumor hypoxia by decreasing oxygen consumption by tumor cells and by increasing oxygen delivery by the tumor vasculature. Human cancer cells treated with NaHS exhibited a decreased OCR that was dose and pH-dependent. The potentiating effect of an acidic pHe on OCR inhibition by NaHS is of particular interest, as hypoxic areas are generally associated with low pH due to cellular adaptations (28, 34) so that pHe values as low as 6.5 have been observed in human tumors (35). Several mechanisms may be implicated in the enhanced inhibitory effect of NaHS on OCR at low pHe. First, as H_2S dissociates to form $HS^- + H^+$ ions with a pKa close to 7, acidosis shifts the balance to the uncharged (H_2S) form, which is permeant to the cell membrane (25). Also, Nicholls and Kim have demonstrated that cytochrome C oxidase inhibition by H_2S is pH-dependent (Ki values ranging from 2.6 µmol/L to 0.07 µmol/L at pH of 8.05 to 6.28, respectively; ref. 36). We also observed that when incubated in the same experimental conditions, OCR inhibition by NaHS was more efficient in MDA-MB-231 cancer cells than in SiHa cancer cells. *In vivo*, a major increase in pO_2 was also found in MDA-MB-231 tumors following NaHS treatment. These different sensitivities may involve the capacity of cells to metabolize H_2S. It has been mathematically demonstrated (37) and experimentally validated (38) that targeting oxygen consumption was the most effective way to reduce tumor hypoxia. Therefore, the different cellular response observed in OCR experiments may account for the greater increased pO_2 found in MDA-MB-231 tumors following NaHS injection.

We also studied the effect of NaHS on tumor perfusion. At first glance, as H_2S induces vasorelaxation in numerous types of blood vessels (3), it would appear that a systemic administration of H_2S would lead to a negative response in tumor perfusion because of the so-called "steal effect" (39). However, our results showed that NaHS injected in the systemic circulation of the mice increased tumor perfusion in two tumor models. The fact that the vasoactive effects of H_2S are oxygen-dependent may play a beneficial role on the vascular response of hypoxic tumors. Indeed, both chronic and intermittent hypoxia increase the expression of K_{ATP} channels (40), the principal vascular target of

H_2S (3). Moreover, it has been reported that H_2S induces vasorelaxation much faster at below physiologic O_2 levels (41).

As we showed that NaHS increases tumor pO_2 by targeting both cancer cells metabolism and tumor perfusion, we then conducted radiosensitizing experiments in the MDA-MB-231 tumor model to test the therapeutic value of the use of NaHS in combination with radiotherapy. There was a significantly increased radioresponse of the tumors when irradiation was applied 15 minutes after NaHS injection, time at which tumor reoxygenation occurred. Confirming that the oxygen level is an important factor for radiosensitization by the H_2S donor, tumors that were clamped during the irradiation were not radiosensitized.

One area related to H_2S treatment for cancer that has already been studied is the effect on proliferation and survival. Changes in the expression of endogenous H_2S-producing enzymes (11) or exogenous administration of H_2S donors (8–10) suggest that H_2S controls tumor progression. Here, we studied the impact of pHe on the cellular response to prolonged exposure to NaHS. At pHe of 7.4, the H_2S donor increased glucose consumption. Enhanced glucose uptake was likely induced in cancer cells to compensate the ATP depletion due to the mitochondrial inhibition observed in these experimental conditions. Because enhanced glucose metabolism is known to promote cancer cell proliferation (28, 29), increased glycolysis by NaHS could potentially account for the increased DNA synthesis rate observed in our study. On the other hand, as H_2S also impaired pHi homeostasis, NaHS exhibited antiproliferative effects when cells were incubated at lower pHe. Taken together, our results emphasize how external factors influence cell response to H_2S.

The chronic injection of NaHS in mice did not provide significant effect on tumor growth, probably because of the microenvironmental heterogeneities characteristic of solid tumors, such as pH gradients. Using xenografts of leukemia cells in mice, others have evidenced the efficacy of a synthetic H_2S donor to restrain tumor growth (10). On the contrary, silencing of the H_2S-producing enzyme cystathionine-β-lyase decreased tumor growth (11). Further experiments with increasing dose of NaHS injected more repeatedly or intratumorally may highlight pro- or anticancer properties *in vivo*. Our experiment suggested a good tolerance of the mice to daily 100 µmol/kg NaHS administration, but more accurate monitoring of *in vivo* toxicity should be considered, especially in dose escalation experiments. Finally, as we showed that the antiproliferative effect of NaHS arises at low pH, more advanced tumors may be more sensitive to the treatment.

In conclusion, we report that NaHS, a fast H_2S-releasing donor, enhances radiotherapy efficacy by alleviating hypoxia in solid tumors. The good tolerance of the mice to chronic NaHS administration further pleads in favor of preclinical evaluation of the combination of H_2S donors to fractionated radiotherapy. When considering H_2S as single treatment for cancer, we report paradoxical effects of H_2S on cancer cell proliferation depending on external pH and no therapeutic benefits *in vivo*. Therefore, we suggest a new approach for the use of H_2S donors in combination therapy

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: P. Sonveaux, B. Gallez

Development of methodology: G. De Preter, C. Deriemaeker, P. Danhier, L. Brisson, P. Sonveaux, B. Gallez

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G. De Preter, C. Deriemaeker, P. Danhier, T.T. Cao Pham

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G. De Preter, C. Deriemaeker, P. Danhier, P. Sonveaux, B. Gallez

Writing, review, and/or revision of the manuscript: G. De Preter, P. Danhier, P. Sonveaux, B. Gallez

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G. De Preter

Study supervision: V. Grégoire, B.F. Jordan, P. Sonveaux, B. Gallez

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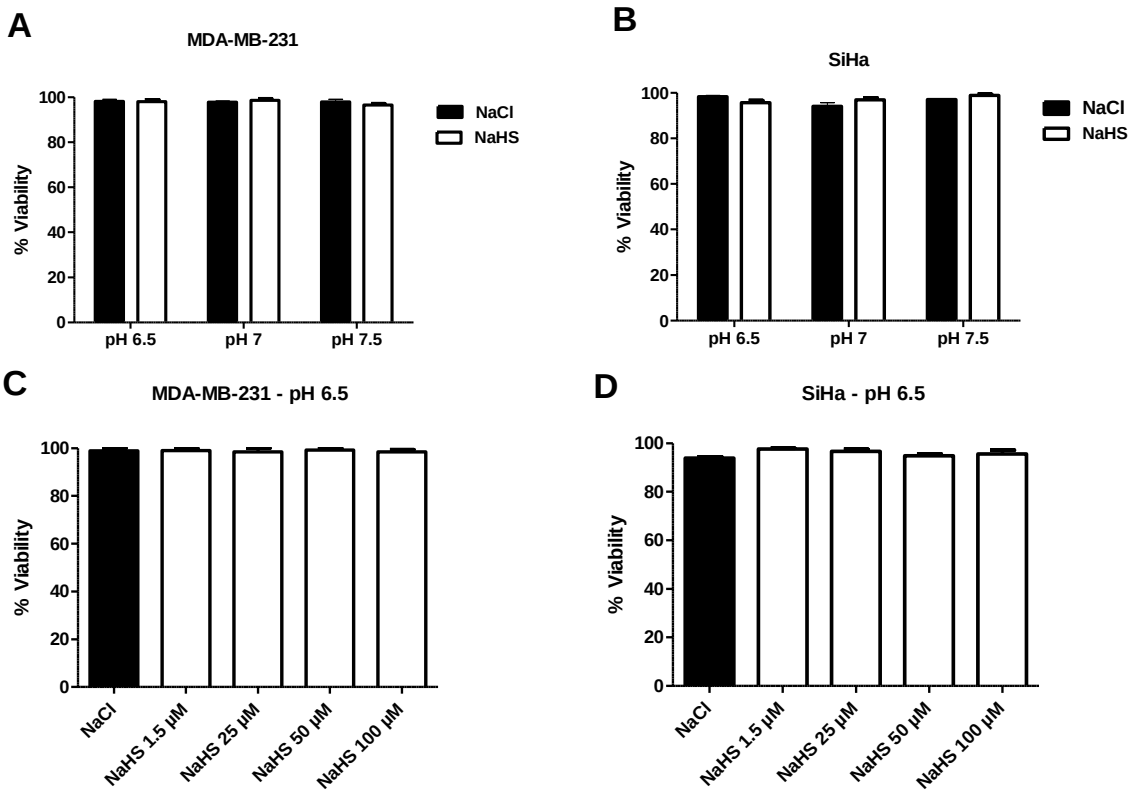
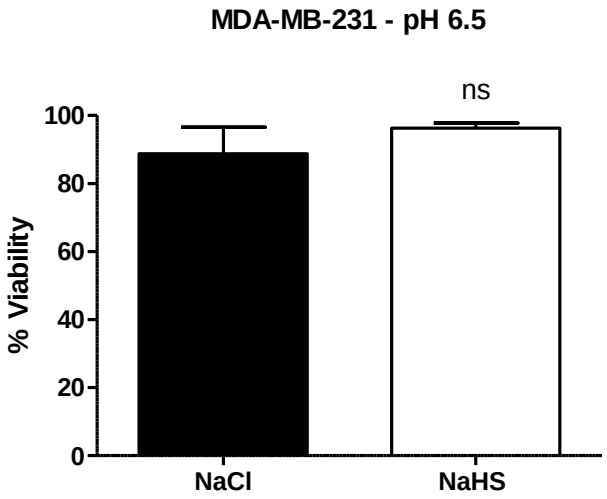
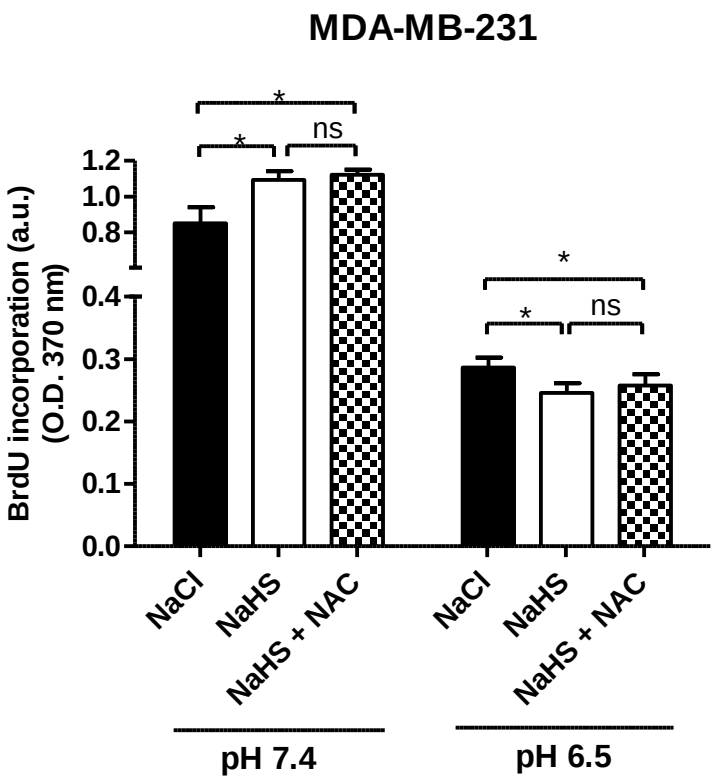
Supplemental Information



Supplemental Fig. S1. Effect of the experimental conditions on cancer cells viability after OCR measurements. Viability of MDA-MB-231 and SiHa cancer cells was measured *in vitro* using trypan blue exclusion after OCR measurements. (A-B) Cancer cells treated with 50 µM NaHS or vehicle (NaCl 0.9 %) in the presence of different pHe values. (C-D) Cancer cells treated with increasing NaHS concentration or vehicle in the presence of pHe = 6.5. No significant cell death was found in any of the experimental conditions.



Supplemental Fig. S2. Effect of the experimental conditions on cancer cells viability after proliferation measurements. Viability of MDA-MB-231 was measured *in vitro* using trypan blue exclusion after BrdU incorporation measurements. Cancer cells were treated with 50 µM NaHS or vehicle (NaCl 0.9 %) in the presence of pHe = 6.5. No significant cell death was found in any of the experimental conditions.



Supplemental Fig. S3. Implication of ROS in the proliferative effects of NaHS. MDA-MB-231 cancer cells were incubated with vehicle (NaCl 0.9 %), 50 µM NaHS alone or pre-treated with 3 mM N-acetyl cysteine (NAC, a ROS scavenger) before NaHS exposure in the presence of different pHe values during 4 hours. Proliferation rates were compared using BrdU incorporation measurements. No change was observed when cells were treated with NAC, suggesting that ROS were not implicated in the pro or anti-proliferative effects induced by NaHS.

Chapter 5 Discussion

Principal findings

We first sought to study the links between glucose metabolism, intracellular ATP content and the proliferation capacity in several cancer cell lines. Using biochemical methods allowing measurements of glycolysis efficiency and mitochondrial respiration, different metabolic profiles were drawn. By correlating the metabolic parameters with intracellular ATP content and DNA synthesis rate, we found that enhanced glycolysis is positively correlated to DNA synthesis. No significant correlation between glycolysis and ATP levels was found. We rather observed a negative trend between glycolysis efficiency and ATP. These results obtained using six different human and murine cancer cell types experimentally showed that glycolysis is a key factor directly contributing to cell proliferation, and confirmed the current hypothesis that its main role may not be dedicated to ATP production. In other words, this observation implies that other factors than ATP production are limiting for proliferating cancer cells. This was also showed by the use of mitochondria-deficient isogenic cancer cells exhibiting a glycolytic switch, associated with an increased DNA synthesis rate and a decreased total ATP pool.

We therefore addressed the role of the PPP in linking enhanced glycolysis and proliferation in two human cancer cell lines. Experiments with a pharmacological inhibitor of the PPP suggested that this pathway is essential for optimal proliferation, especially in glycolytic cancer cells. This was confirmed with siRNAs targeting G6PD, the first and rate-limiting enzyme of the oxidative branch of the PPP.

We finally identified that DCA, a promising molecule for the treatment of malignant lesions, decreases cancer cell proliferation in a glycolysis-dependent manner.

Altogether, we propose that a reduction in the glycolytic metabolism of glucose induced by DCA also decreases the PPP flux along with cellular proliferation.

A second part of the thesis focused on a different approach to target metabolism for cancer therapy. Inhibition of mitochondrial respiration by H_2S was hypothesized to increase the sensitivity of tumors to radiotherapy. Indeed, mitochondrial ETC complex IV is known as the primary target of H_2S, and inhibition of O_2 consumption appears to be a particularly effective strategy to alleviate hypoxia, a serious impediment in radiation efficacy. *In vitro*, we found that NaHS, a H_2S donor, decreased mitochondrial respiration in a dose- and pH-dependent manner, which was associated with an increased glycolytic flux. Enhanced perfusion following NaHS delivery was also observed *in vivo*. Using electron paramagnetic resonance (EPR) oximetry, we reported a rapid increase in pO_2 after NaHS administration in two human tumor models in mice. Furthermore, tumors irradiated after a single NaHS administration were more sensitive to irradiation. Radiosensitization was due to an oxygen effect as the increased growth delay was abolished when temporarily clamped tumors were irradiated. We finally aimed to investigate whether the H_2S donor could have a direct effect on tumor growth. Chronic NaHS injections showed no significant impact on tumor growth curves in mice. We highlighted that pHe induced paradoxical responses to NaHS. An increased proliferation was found at alkaline pHe whereas a decreased proliferation was found at acidic pHe following exprosure of cancer cells to NaHS. This may, at least in part, explain why NaHS was found inactive *in vivo.* A summary of the findings on the use of the H_2S donor in combination with radiotherapy or in monotherapy is provided in figure 13.

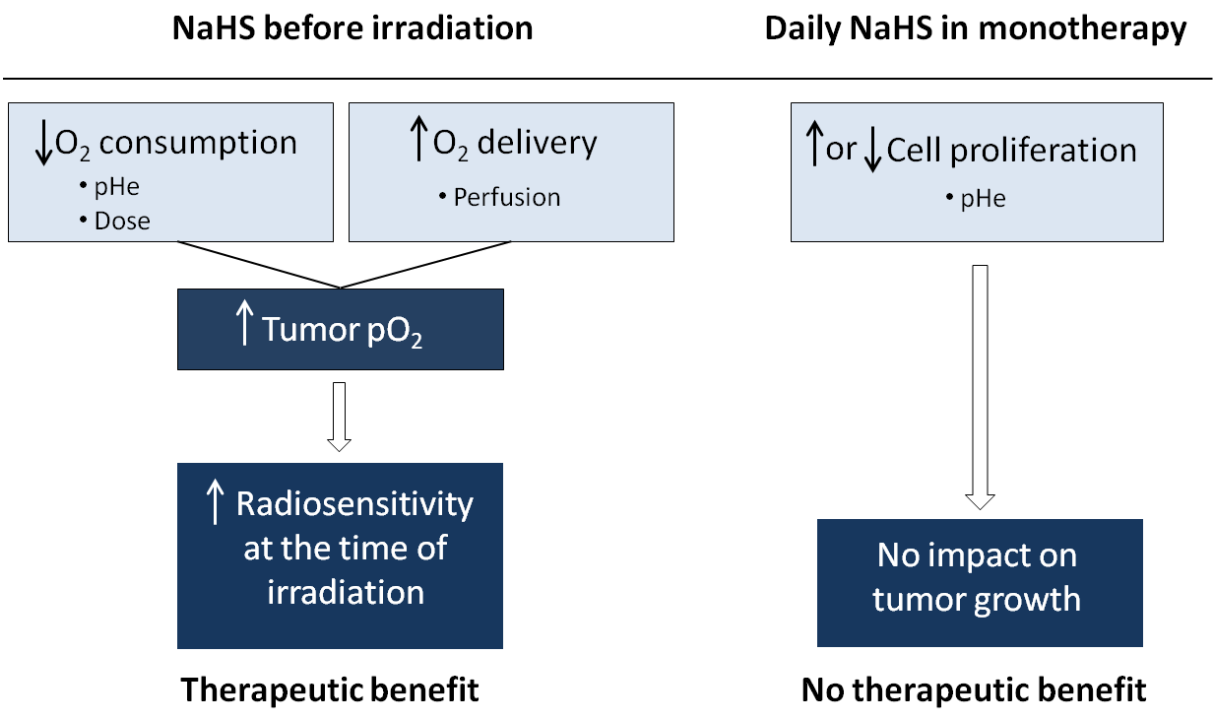


Figure 13: Principal findings obtained from the use of NaHS, a fast H_2S-releasing donor, in our study. NaHS administration a few minutes before irradiation provided therapeutic benefit by alleviating tumor hypoxia. We suggested that the improved tumor oxygenation by H_2S derived from NaHS at the time of irradiation resulted from an inhibition of the oxygen consumption by tumor cells and an increase in tumor perfusion. We highlighted that the inhibitory effect of NaHS on oxygen consumption was pHe- and dose-dependent. On the other hand, daily NaHS injection was found ineffective to control tumor xenografts in mice. Indeed, *in vitro*, it was found that NaHS induced opposite effects on tumor cell proliferation depending on the pHe.

Discussion and Perspectives

1. Glycolysis drives cancer cell proliferation

1.1 *In vitro* characterization of bioenergetics and proliferation: limitations and perspectives

Experiments that aimed to evaluate the metabolic parameters (glycolysis and OXPHOS activities), energy stores and proliferation capacities of cancer cells were performed separately. Indeed, technical procedures did not allowed concomitant measurements of all parameters. As examples, cell harvesting, cell lysis, and/or cell fixation were required depending on the experiment. It should therefore be noted that the multiple correlations between metabolic and proliferative patterns could thereby suffer from inevitable experimental bias. Concomitant analysis of the patterns at the same moment, in the same viable cells, would appear ideal. In fact, the most difficult would be to quantify intracellular ATP, which systematically includes extraction processes. Interestingly, a new technique that allows the rapid, direct measurement of the intracellular ATP content in living cells was reported. This original system relies on a protein transduction domain-conjugated luciferase (PTD-Luc) that can penetrate the cell membrane without affecting its integrity or interfering with cellular metabolism *(Lee et al., 2012)*.

In this study, the efficacy of the conversion of glucose to lactate was used to compare the glycolytic activity between cell lines. This method offers the advantage to more closely reflect the glycolytic flux as compared to molecular biology approaches. Variation in mRNA content or protein expression is not always proportional to changes in enzyme/transporter activity, and much less to changes in metabolic flux *(Moreno-Sanchez et al., 2014)*. Nevertheless, a significant limitation to the enzymatic determination of lactate/glucose ratio can be highlighted. Glucose is

the major source of lactate, but glutaminolysis may also significantly contribute to lactate production in cancer cells (Chapter 1 III.2). In our experimental conditions, metabolite analysis was performed on cells incubated for 24 hours without glutamine. Therefore, the contribution of glutamine to lactate production was considered to be negligible. It can be mentioned that the specific determination of the different sources of lactate can be achieved using isotope-labeling approaches that are more accurate to evaluate the fate of nutrients.

1.2 Inhibition of the pentose phosphate pathway: implicated mechanisms and therapeutic potential

The PPP is a glucose catabolic pathway that links glucose metabolism to nucleotide biosynthesis and NADPH production. This process provides two significant factors for tumor progression: **biosynthetic precursors** through the generation of R-5-P (nucleotide precursor) and NADPH (reductive equivalent for macromolecule synthesis) and **antioxidant protection**, involving the transfer of electrons from NADPH to thioredoxin/peroxiredoxin and glutathione systems.

In our study, inhibition of the PPP was found following treatment of cancer cells with 6-aminonicotinamide (6-AN), siRNAs against G6PD and DCA. All treatments were associated with a reduced proliferation capacity in cancer cells. It would have been interesting to further investigate whether one (or both) of the above-mentioned mechanisms is (are) predominantly implicated in the anti-proliferative effects of these treatments. In particular, this would have allowed to precisely understand how DCA affects cancer cell proliferation. Indeed, DCA when used at similar doses as the ones that we used in our study (5 mM) has also been described to induce ROS production *(Stockwin et al., 2010)*. Decreased PPP flux by DCA could therefore account for a decreased biosynthetic capacity and/or a decreased protection against ROS. To investigate whether a reduction in R-5-P synthesis is

responsible for the inhibition of cancer cell proliferation by DCA, supplementation of culture media with nucleosides and ribonucleosides could have been tested *(Frolova et al., 2011)*. Rescue using downstream products of the PPP would have further provided evidence that it is the nucleotide precursor shortage and not lower NADPH levels that is responsible for decreased cell proliferation. Conversely, incubation of cells with antioxidants would have underlined the antioxidant role of the PPP.

While numerous glycolytic inhibitors are being investigated in humans, there is currently no inhibitor of the PPP in clinical trials, even though the PPP may be an attractive target. 6-AN has already been evaluated as an anticancer compound but was found to be neurotoxic at high doses and failed to demonstrate anticancer activity in well tolerated doses *(Herter et al., 1961)*. 6-AN still remains under consideration as a potential radiosensitizer *(Sharma et al., 2012; Manganelli et al., 2013)*. Inhibition of the PPP or of G6PD may also enhance the sensitivity of cancer cells to chemotherapeutic agents known to induce genetic damage and to produce ROS *(Jones and Schulze, 2012)*. A very interesting study screened small-molecule compound libraries and identified new human G6PD inhibitors with IC50 values < 4 µM. One of the inhibitors reduced the proliferation of mammary carcinoma cells *(Preuss et al., 2013)*. This will probably arouse the interest for the use of specific PPP inhibitors alone or as co-treatment in clinical cancer research.

2. Reversing the Warburg Effect with dichloroacetate

2.1 Metabolic plasticity in MDA-MB-231 and SiHa cancer cells

The Warburg hypothesis on the origin of cancer has been revisited and the environment of cancer cells (nutrients, O_2, pH, hormones, drugs…) is now well recognized to play a determining role on tumor bioenergetics. By showing that DCA increases glucose oxidation (which only occurs in functional mitochondria) in lung, brain and mammary cancer cells, Bonnet and colleagues *(Bonnet et al., 2007)* provided evidence that aerobic glycolysis, a well-established, metabolic signature of many cancer cells, is most often reversible rather than a general consequence of permanent mitochondrial damage.

Our group studied the glucose consumption of MDA-MB-231 and SiHa cancer cells under hypoxia (1% O_2) and normoxia (21 % O_2) in order to investigate the metabolic plasticity of these cells. MDA-MB-231 cancer cells consumed glucose at a higher rate than SiHa in hypoxia, but both cell lines showed a decreased glucose consumption when the O_2 supply was increased (unpublished data); suggesting a Pasteur Effect even in highly glycolytic cells. In another study, our group also showed that increased oxygenation induced by carbogen breathing in MDA-MB-231 and SiHa tumor xenografts led to a decreased uptake of ^{18}F-FDG (^{18}F fluorodeoxyglucose), a glucose analog used in positron emission tomography (PET) studies, in both tumor models *(Neveu et al., 2015)*. These observations suggest that, in our tumor models, glycolysis can also be altered by promoting OXPHOS.

2.2 Differential response to DCA

Preclinical studies with DCA have shown variable responses between cancer cells (Chapter 1 II.3.2). This emphasizes the need for biomarkers that can predict the therapeutic response to PDK inhibitors. Both cancer cells lines used here

(MDA-MB-231 and SiHa) can remodel their glucose metabolism in response to external stimuli, thus displaying metabolic flexibility. However, short-term lactate production measurements showed that DCA decreased glycolysis in MDA-MB-231 cancer cells. This resulted in a decreased proliferation in this cell line compared to SiHa. The isogenic comparison of oxidative SiHa cancer cells *versus* SiHa with a high glycolytic activity further validated that glycolytic cells are more sensitive to DCA than oxidative ones. These findings are in agreement with a recent study showing that glycolytic, metastatic prostate cancer cells are DCA-responsive, whereas their isogenic less glycolytic counterparts are unresponsive *(Kailavasan et al., 2014)*. It also supports that the activity of DCA is enhanced when glycolysis is upregulated in cancer cells *(Stockwin et al., 2010)*. Nonetheless, it should be highlighted that observations from another study led to the opposite conclusion *(Higgins et al., 2009)*.

One could simply suggest that the anti-proliferative effects of DCA are directly related to the variable reliance of cells on glycolysis-branched anabolic processes. It is our opinion that it is not the primary cause of the different responses to DCA. Because short-term evaluation (1 hour) of DCA efficacy showed that DCA inhibits glycolysis in responsive cells but not in resistant ones, we rather suggest that the primary factor in DCA efficacy is its capability to efficiently inhibit PDK, resulting in tumor cell metabolism rewiring and altered cell proliferation. Some hypotheses can be put forward to try understanding how cellular singularities, sometimes linked to the metabolic profile, can influence the response to DCA:

- Access to intramitochondrial PDK

Dichloroacetate is ionized and cannot pass through the plasma membrane by diffusion. To the best of our knowledge, there have been only two reports on the transport of dichloroacetate into mammalian cells. The first one identified that monocarboxylate transporters (MCTs) facilitate the uptake of DCA into hepatocytes

and Ehrlich-Lettre tumor cells *(Jackson and Halestrap, 1996)*. The second one showed that the sodium-coupled MCT (SMCT) SLC5A8 transports DCA very effectively with a higher affinity compared to MCTs *(Babu et al., 2011)*. This transporter is sometimes found to be silenced in tumor cells via epigenetic mechanisms, which was suggested to explain why high millimolar concentrations of DCA are needed to cause a therapeutic response *in vitro.* We could therefore hypothesize that the differential sensitivities of cancer cells to DCA could be related to their relative expression of MCTs, and, especially, of SLC5A8, and that evaluation of the differential protein expression profiles in glycolytic and oxidative cancer cells may be relevant. In this regard, it has already been reported that the expression of MCTs differs depending on the metabolic phenotype of cancer cells *(Sonveaux et al., 2008)*. Although not yet investigated, the level of expression of mitochondrial pyruvate carriers (MPCs) may also impact the response of cells to DCA. MPCs could indeed determine the rate of penetration of DCA inside the mitochondrial matrix *(Schell et al., 2014)*.

- PDK isoforms

The four PDK isozymes vary considerably in their sensitivity to inhibition by DCA. PDK2 is the most sensitive, PDK3 the most resistant, and the sensitivities of PDK1 and PDK4 are intermediate. On the other hand, each PDK isoform has its own specific activity. Most active isoenzyme PDK3 is 25-fold more active than the least active PDK2 *(Bowker-Kinley et al., 1998)*. Therefore, a low affinity of DCA for PDK3 may be sufficient to alleviate PDH inhibition because of the high potency of PDK3 to impair PDH function. In this regard, analysis of the expression pattern of PDK isoforms between cell lines may be informative of the response to DCA, but specific activities should not be underestimated.

- DCA biotransformation

DCA is eliminated mainly through glutathione transferase zeta 1 (GSTZ1)-catalyzed dechlorination to glyoxylate, an intermediate in the biotransformation of DCA to oxalate, CO_2, and glycine *(Li et al., 2011)*. Many cancer cell lines do express GSTZ1, including SiHa, at relatively different levels (www.proteinatlas.org)[1]. Correlating GSTZ1 expression and DCA efficacy between cancer cells could give new insights on the role of DCA elimination in the therapeutic response. Because GSTZ1 catalyzes DCA biotransformation through a glutathione (GSH)-dependent mechanism *(Board and Anders, 2005)*, comparison of GSH levels between cancer cells may also be relevant.

2.3 To what extend can these *in vitro* findings anticipate the *in vivo* situation?

In vitro analyses of cancer cell metabolism undoubtedly help demystifying its complex implications in cell proliferation and survival. It considerably helps identifying specific pathways or targets for future therapeutic approaches. It also contributes to the comprehension of drug resistance. However, it is clear that *in vitro* and *in vivo* environments have little in common. Compared to the artificial culture conditions, tumors experience constant changes in many factors, such as oxygenation, pHe, and nutrients availability.

In vitro, we have shown that low millimolar concentrations of DCA decrease glycolysis along with the PPP flux, thereby limiting cancer cell proliferation. It should be mentioned that, even if glucose seems preferentially used as a source of energy and building blocks by tumor cells, glutamine and fatty acids can also significantly contribute to tumor growth. Consequently, inhibition of glycolysis by DCA could potentially be compensated by these alternative sources (metabolic flexibility).

[1] Web site visited on November the 4th, 2015

Moreover, expression and activity of PDK isoforms are sensitive to environmental stimuli. Metabolic stress conditions, such as starvation, fasting, and glucose deprivation can, for example, induce PDK4 expression *(Hsieh et al., 2008)*. Therefore, it is difficult to anticipate whether our *in vitro* observations, obtained in artificial culture conditions with glucose as main source of energy, are also found *in vivo*.

Human cancers also frequently display substantial intra-tumor heterogeneity in virtually all phenotypic features, including bioenergetics *(Marusyk and Polyak, 2010)*. As different metabolic profiles can be exhibited by different tumor cells subpopulations, some tumor cells (oxidative) may be resistant while others (glycolytic) may be sensitive to DCA. Efficacy of DCA *in vivo* may therefore depend on the relative prevalence of oxidative vs. glycolytic cells within a given tumor.

To study the metabolic and proliferative effects of DCA at clinically relevant doses *in vivo*, sensitive methods are required. Hyperpolarized nuclear magnetic resonance (NMR) tracers can increase the sensitivity of ^{13}C magnetic resonance spectroscopy and allow non-invasive and real-time assessment of metabolic fluxes *in vivo*. Increasing the polarization of a particular compound, which determines the strength of the MR signal, can be achieved by dynamic nuclear polarization (DNP). This technique consists in the transfer of high electron spin polarization to nuclear spins via microwave irradiation in a strong magnetic field and at cryogenic temperature *(Schroeder et al., 2011; Mignion et al., 2014)*. Recently, glycolysis in murine lymphoma and lung tumors was monitored by measuring the conversion of hyperpolarized glucose to lactate *(Rodrigues et al., 2014)*. Production of 6-phosphogluconate (6-PG) in the oxidative branch of the PPP was also detected suggesting that PPP evaluation is feasible with this method. The use of

hyperpolarized NMR tracers would therefore be of great utility to investigate whether low dose DCA (≤ 25 mg/kg orally, as tolerated in patients) interfere with glycolysis and PPP activities in mice. Indeed, such DCA concentrations may induce relatively small changes in metabolic fluxes. On another hand, PET in association with thymidine-analog tracers would allow studying changes in DNA synthesis in response to DCA. Early clinical data with ^{18}F-FLT (^{18}F-labeled 3'- deoxy-3'-fluorothymidine) demonstrated that its uptake correlates well with *in vitro* measurements of proliferation *(Bading and Shields, 2008)*.

2.4 Perspectives on the clinical use of DCA in cancer therapy

When analyzing published results pertaining to clinical trials of oral DCA in cancer patients, DCA has proven encouraging in terms of tumor stabilization. However, like with other glycolysis inhibitors, chronic DCA treatment was associated with toxicity in several patients (Chapter 1 II.3.2.2). A major issue is dose-dependent neuropathy caused by oxidative stress in the peripheral nerves. For optimal therapeutic benefits, markers of response should be investigated. This would allow selecting responsive patients. We and others have highlighted that glycolytic cancer cells may be more sensitive to DCA. Intensity of incorporation of ^{18}F-FDG, which is already used for diagnostic and monitoring purposes in the clinics, could therefore be indicative of the response to DCA *in vivo*, even though the degree of utilization of glycolysis and OXPHOS by cancer cells cannot be evaluated with this technique alone. Accurate and sensitive methods allowing the determination of predominant energetic pathways within a tumor are still lacking, and implementation of such methods in the clinics would undoubtedly help choosing the adequate therapeutic approach for each patient.

Preclinical studies have linked the sensitivity of cancer cells to DCA to the presence of specific transporters and PDK isoforms. These findings should also be considered.

Like other groups previously, we have shown that low millimolar concentrations of DCA reduce cancer cell proliferation without altering cell survival. Because cytostatic effects may not be sufficient to provide a significant therapeutic gain or because they may be balanced by compensatory processes, we support the current view consisting in the use of DCA in combination therapy. One promising association is DCA and metformin. Metformin has been used for a long time as antidiabetic drug and has more recently been shown to display anticancer effects by targeting mitochondrial ETC complex I. Preclinical data are available and suggest that a co-treatment with DCA and metformin results in synergistic anticancer effects *(Choi and Lim, 2014; Haugrud et al., 2014)*

3. H_2S as a new radiosensitizer

3.1 Effects of NaHS on the oxygen consumption of cancer cells: a need for *in vivo* validation

Our *in vitro* experiments emphasized that O_2 consumption inhibition by H_2S is pH-dependent, which is of major importance regarding the fact that acidic regions are commonly found in solid tumors. Thus, NaHS could more potentially target O_2 consumption in tumors than in healthy tissues. Nevertheless, we also showed that a sufficient NaHS concentration (50 µM) was needed to induce significant effects. Further studies on O_2 consumption *in vivo* are therefore mandatory. It would unequivocally provide evidence that a decreased O_2 consumption by tumor cells is implicated in the observed increase in pO_2 following systemic NaHS injection (100 µmol/kg I.P.). Hence, comparison with healthy tissues, such as adjacent muscle tissue, could confirm the expected tumoral selectivity of H_2S.

Intratumoral H_2S concentration could also be measured to test whether sufficient H_2S is present to inhibit O_2 consumption. Low H_2S concentrations (< 10 µM) are known to increase mitochondrial oxidative activity, which could lead to unfavorable pO_2 variations. Obtaining accurate and reliable measurements of biologically free H_2S is laborious. Sulfide ion selective electrodes have however shown efficacy to detect H_2S levels in biological samples, but they may suffer from limited sensitivity. H_2S can be more sensitively measured by reversed-phase high liquid performance chromatography (RP-HPLC) coupled with fluorescence detection. This method, which requires sulfide derivatization, has been suggested to be suitable for accurate quantitative measurement of free H_2S in multiple biological samples *(Shen et al., 2011)*.

3.2 NO and H_2S: two radiosensitizing molecules with similar and selective modes of action in tumors

Our group has previously contributed to the demonstration that NO can radiosensitize solid tumors through different mechanisms (Chapter 1 III.3.3). We now provide a proof of concept that H_2S, the last identified endogenous gasotransmitter to date, shares this therapeutic potential. Due to the fact that NO and H_2S have various similar properties, it appears important to compare their radiosensitizing effects (Table 2).

Interestingly, increasing evidence suggests that H_2S and NO may reciprocally regulate their production and biological effects. H_2S donors were indeed reported to induce eNOS activity through phosphorylation processes, subsequently increasing NO production *(Kolluru et al., 2013)*. Reciprocally, NO donors can up-regulate the expression and activity of H_2S-producing enzymes. Interestingly, potential synergistic effects of NO and H_2S in controlling the vascular function have also been highlighted *(Hosoki et al., 1997)*. H_2S/NO relationships are complex and still poorly defined, especially in the context of tumors, but one cannot exclude that H_2S enhances the radioresponse through NO-dependent pathways, and *vice-versa*.

It must be finally added that effects of NaHS on the cell cycle may also contribute to the observed radiosensitization process. Indeed, cell cycle phases can influence cellular responses to radiations *(Sinclair, 1968)*. In published studies, H_2S has been reported to induce a cell cycle arrest in S or G2/M phases depending on its mode of administration and concentration *(Cai et al., 2010; Lee et al., 2011)*.

	NO	H_2S	Remarks
Radio-sensitizing effects	***O_2-dependent*** ↓ O_2 consumption ↑ perfusion ***Intrinsic*** Yes	***O_2-dependent*** ↓ O_2 consumption ↑ perfusion ***Intrinsic*** No	• While mitochondrial cytochrome C oxidase reversible inhibition is described for both molecules, vasoactive effects implies different mechanisms. • There is evidence that NO intrinsically improves stabilization of radiation-induced DNA damage. Conversely, H_2S has been reported to have intrinsic radioresistant effects.
Intrinsic tumor selectivity	ND	Yes	• This study showed that oxygen consumption inhibition by H_2S is enhanced in acidic conditions. Moreover, expression of H_2S vascular target (K_{ATP} channel) is increased by hypoxia . No tumor selectivity has been reported for NO itself, but selective NO delivery can be achieved with the use of carriers which release NO in hypoxic or acidic environments.
Mode of Administration	***NO donor*** isosorbide dinitrate, xanthinol nicotinate, S-nitrosocaptopril ***Stimulation of endogenous NO production*** insulin, electrical pulses	***H_2S donor*** Sodium hydrosulfide	• Mode of NO administration influences the mechanisms implicated in the radiosensitization process.

Table 2: Comparison of the radiosensitizing effects, intrinsic tumor selectivity and mode of administration of NO and H_2S.

3.3 Clinical applicability of H_2S in cancer therapy

Safety of inhaling H_2S gas is a key issue, and inhaled H_2S has been associated with industrial and environmental risk *(Wang, 2012)*. Inhalation of H_2S has however been attempted in animal studies to induce suspended animation, a hibernation-like state. Indeed, in their pioneering study, Blackstone and colleagues *(Blackstone et al., 2005)* showed that awaken mice exposed to sub-toxic gaseous H_2S concentrations (20-80 ppm) dose-dependently decreased their O_2 consumption. This fall in metabolic activity was associated with bradypnea and consecutive hypothermia. Upon H_2S removal, these effects were completely reversed and animal functions rapidly returned to normal. Several differences between smaller and bigger animals may however considerably influence the hypometabolic responses to H_2S. First, difference in surface area-to-mass ratios may render larger animals more resistant to temperature changes. Second, higher H_2S concentrations may be required to induce a hypometabolism in larger animals and in humans *(Wang, 2012)*. This approach could be problematic or even dangerous considering the toxicological profile of H_2S at high concentrations. Therefore, inhalation of H_2S gas has not been clinically tested yet.

Comparatively, injectable H_2S-releasing molecule could be more safe and suitable to treat cancer, especially with radiotherapy. In particular, fast and high H_2S release is preferred. Indeed, increased tumor oxygenation is required only at the time of irradiation. In addition, regarding the paradoxical effects of the gas on mitochondrial ETC depending on local concentration, high H_2S delivery is needed. It is important to note that sodium sulfide (Na_2S), a H_2S-releasing inorganic salt similar to NaHS, has already been evaluated in patients. In a clinical trial, it was aimed to determine whether IK-1001 (Na_2S administered as an isotonic solution) was safe and if it could reduce damage caused by severe types of heart attacks (NCT01007461).

Unfortunately, this study has been withdrawn prior to enrollment in 2015. Withdrawal was due to company decision but was not safety-related. Another phase 1 study was launched to assess the pharmacokinetics of IK-1001 in healthy volunteers as well as in subjects with varying degrees of impaired renal function (NCT00879645). This study was interrupted in 2015 because of the inability to develop a rapid and reliable assay to detect sulfide concentration.

A group recently reported a promising strategy for targeted H_2S delivery to the heart in order to limit myocardial ischemia-reperfusion injury *(Chen et al., 2014)*. This novel approach is based on H_2S-filled microbubbles that, when injected systematically, are confined to the vasculature due to their size. With judicious application of ultrasound (US) energy, H_2S can then be efficiently delivered to the targeted tissue through the process of sonoporation. Microbubble ultrasound contrast agents (MCA's) are becoming increasingly popular for targeting drugs and genes to malignant tissues *(Sirsi and Borden, 2012)*. H_2S-filled microbubbles are thus promising as they could potentially reduce off-target effects and increase the therapeutic drug index of H_2S.

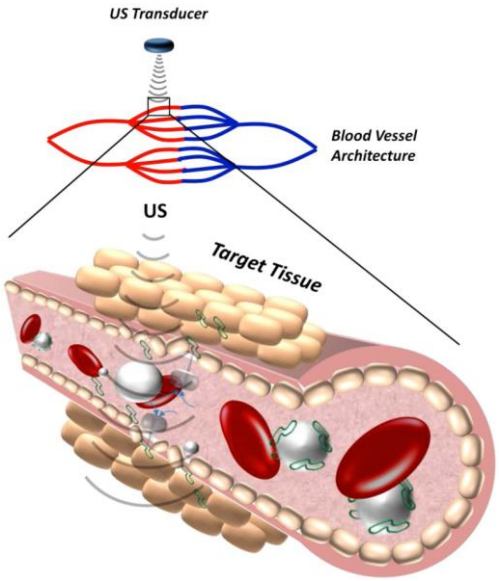


Figure 14: Microbubble ultrasound contrast agents (MCA's) injected in the blood circulation are confined into the vasculature. The physical response of microbubbles to an ultrasound field can mechanically perturb blood vessel walls and cell membranes, enhancing drug permeability into tissues, such as malignant tissues.

Sirsi and Borden, 2012.

4. General conclusion

The present study emphasized the therapeutic potential of targeting cancer cell metabolism. We showed that compounds inhibiting either aerobic glycolysis or mitochondrial respiration provided therapeutic gain. We highlighted that metabolism-targeting compounds may often benefit from the association with other therapies. Indeed, inhibition of cancer cell proliferation with tolerable DCA concentrations may have limited efficacy in patients. Moreover, this effect may be compensated by tumor cells metabolic plasticity. H_2S, a mitochondrial inhibitor with multiple biological functions was found ineffective to treat tumors in mice, but enhanced tumor growth delay when associated with radiotherapy. In terms of toxicity and efficacy, further preclinical and clinical research on the inhibition of metabolic pathways in combination therapy would therefore likely provide significant advances for optimal cancer management.

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