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Bioenergetic pathways in tumor mitochondria as targets for cancer therapy and the importance of the ROS-induced apoptotic trigger.

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Abstract

Mitochondria are emerging as idealized targets for anti-cancer drugs. One reason for this is that although these organelles are inherent to all cells, drugs are being developed that selectively target the mitochondria of malignant cells without adversely affecting those of normal cells. Such anti-cancer drugs destabilise cancer cell mitochondria and these compounds are referred to as mitocans, classified into several groups according to their mode of action and the location or nature of their specific drug targets. Many mitocans selectively interfere with the bioenergetic functions of cancer cell mitochondria, causing major disruptions often associated with ensuing overloads in ROS production leading to the induction of the intrinsic apoptotic pathway. This in-depth review describes the bases for the bioenergetic differences found between normal and cancer cell mitochondria, focussing on those essential changes occurring during malignancy that clinically may provide the most effective targets for mitocan development. A common theme emerging is that mitochondrially mediated ROS activation as a trigger for apoptosis offers a powerful basis for cancer therapy. Continued research in this area is likely to identify increasing numbers of novel agents that should prove highly effective against a variety of cancers with preferential toxicity towards malignant tissue, circumventing tumor resistance to the other more established therapeutic anti-cancer approaches.

Keywords: mitochondria, apoptosis, cancer, bioenergetic pathways, reactive oxygen species (ROS), mitocans.

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1. INTRODUCTION:

Excessive dietary intake leading to overweight physiques and obesity is now recognised as a risk factor linked to numerous co-morbidities including higher incidence of cancers (Renehan et al., 2009; Guh et al., 2009). Perhaps nowhere is this more apparent than with the now well established link between excessive alcohol intake, carcinogenesis and increased cancer incidence (O’Hanlon, 2005). A related question is whether excessive (conspicuous) consumption acts on the cell mitochondria to drive cancer development and growth, and the corollary that mitochondria may be excellent targets for cancer therapy. It is clear that cancer cells survive and thrive by their much greater capacity than normal cells to utilize a broad range of alternative energy substrates. Rapidly growing tumors deplete the available nutrients and this scavenging action of cancers is associated with cachexia and sudden weight loss, a diagnostic hallmark for the disease. However, rapacious growth comes at a price to tumors, particularly within the tumor bulk as cells deplete the available resources, become hypoxic and exceed the blood supply of nutrients.

The cancer cells within tumors deprived of glucose are able to maintain their ability for mitochondrial ATP synthesis through oxidative phosphorylation (OxPhos), or activate a metabolic switch to generate their ATP by this process, through the oxidation of fatty acids and amino acids — catabolic pathways. The biochemical switch towards catabolism, when cellular constituents are degraded to produce energy is critical for the deprived/quiescent cancer cells, providing a temporary survival mechanism until the supply of other nutrients such as glucose is restored. In the deprived state, the mitochondria act as essential organelles providing many basic metabolites and energy to enable cancer cells to maintain their survival and slow expansion, despite the stressful conditions imposed. The residual low or high capacity of mitochondrial function inside cancer cells results from a highly modified bioenergetic metabolism although the cells remain dependent on many of the essential mitochondrial processes without which cells cannot thrive. These modified bioenergetic pathways associated with cancer cell mitochondria are the focus of

this review as is the recognition that they provide novel paradigms for the treatment of cancer, acting as a built-in device to elicit cancer cell apoptosis.

Although much has been published about the significance of anaerobic fermentation (glycolysis) in normoxic conditions by cancers overriding mitochondria as the source of energy substrates (Warburg, 1956), this interpretation has been heavily biased and obscures the contribution of residual or fully active mitochondrial functions. Consequently, the vital roles mitochondria play in cancer cell survival and growth have remained neglected and underestimated. The current review attempts to redress this imbalance and shed more light on Warburg's proposed Origins of Cancer (1956) where he states: "If the respiration falls below a certain minimum that the (cancer) cells need unconditionally, despite their increased fermentation, they die; whereas the normal cells, where respiration may be harmed by the same amount, will survive because, with a greater initial respiration, they will still possess a higher residual respiration...". The highly significant implication of Warburg's statement is that cancer cells likely have a minimum threshold of respiration such that they are vulnerable to further reductions, leading to cell death and as we now know this means by mitochondrial-mediated apoptosis. Therein may lie the Achilles heel of cancer.

2. ALTERNATIVE ENERGY SUBSTRATES FOR CANCER CELLS.

The alternative energy substrates considered in this review, particularly with reference to the role of mitochondria, include contributions of acetyl-CoA *via* citrate for fatty acid synthesis leading to lipid production as well as sources of energy from β -oxidation of fatty acids; small carboxylic acids; ketone bodies; amino acids such as glutamine/glutamate and their anaplerotic entry into the Krebs's cycle and dihydroorotate dehydrogenase catalyzing *de novo* pyrimidine biosynthesis required for DNA replication. The effects of various drugs blocking these alternative sources of energy metabolites used by cancer mitochondria and their impact in killing tumor cells provide greater understanding of the relative contributions of these metabolic pathways and which

are the more critical elements important for cancer cell survival. All of these alternative energy sources involve mitochondrial mediated pathways and result in oxidative stress and production of reactive oxygen species (ROS) which if sufficiently high, can be the trigger for cancer cell apoptosis. Therefore, they may provide excellent targets for cancer therapy.

2.1 Pyruvate and the Pyruvate Dehydrogenase (PDH) complex: one of the the major points of divergence between cancer and normal cells. Role of alcohol, acetaldehyde, acetoin and lipoic acid.

One of the major, if not *the* major point of divergence distinguishing normal from cancer cell metabolism that also reflects the importance of dietary influences, occurs at the end of the glycolytic pathway as modified activity of the pyruvate dehydrogenase (PDH) complex. PDH is one of the most critical enzymes distinguishing cancer cell metabolism on the basis of the two alternative PDH substrates available in cancer cells, namely pyruvate and acetaldehyde (Fig. 1).

Early biochemical studies analysed the relative flow rates of carbon fuel from the glycolytic pathway driving the mitochondrial powerhouses inside cancer cells. Thus, Lazo and Sols (1980) were amongst the first to study PDH complexes isolated from Ehrlich ascites tumors for *in vitro* analyses, revealing that PDH activity was indeed relatively small compared to that from normal liver cells. The ascites tumor PDH activity was only 0.1 unit of enzyme activity/g packed cells by wet weight, whereas in normal tissues such as mammary gland, PDH activity was much higher at close to 1 unit/g tissue wet weight (Coore and Field, 1974; Postius 1978; Lazo and Sols, 1980). Kinetic analysis of ascites tumor PDH showed a similar affinity for pyruvate ($K_m=17-46\ \mu\text{M}$) and NAD^+ ($K_m=110\ \mu\text{M}$) to normal liver, heart and brain PDH ($K_{m_{\text{pyr}}}=28-45\ \mu\text{M}$; $K_{m_{\text{NAD}}}=45-125\ \mu\text{M}$), but lower affinity for CoA (K_m value of 36 vs. 5-9 μM , respectively) (Siess et al., 1976, Lazo and Sols, 1980). Thus, in addition to a diminished expression, the lower tumor PDH activity was also related to its low CoA affinity. While lactate production from glucose via pyruvate was recorded at 4-10 $\mu\text{moles}/\text{min}/\text{g}$ packed cells by wet weight (Brin and

McKee, 1956; Coe et al., 1966), only 5 to 15% of pyruvate could be catabolized by PDH at its full activity in the Ehrlich ascites cancer cells (Seiss et al., 1976). A similar conclusion arises from the results of Weinhouse and Wenner (1956), who compared lactic acid and CO₂ formation from ¹⁴C-glucose with 77-94% of glucose supplied to tumor cells converted to lactic acid. Thus, PDH activity appeared to be a limiting factor in oxidative glucose breakdown by Ehrlich ascites tumor cells and was well below the reported maximal rate of pyruvate transport (5-12 μmol/min/g cell protein) into their mitochondria (Eboli et al., 1977; Paradies et al., 1983). Hence, it was proposed that the restricted PDH activity inside the Ehrlich cancer cells represented a rate limiting point in energy flow. In contrast, in other rat ascites tumor cells such as AS-30D, pyruvate oxidation reaches similar rates to those reported for rat liver mitochondria (Moreno-Sánchez et al., 2007; 2009). Hence, whether PDH represents a rate-limiting step depends on the cancer cell type.

2.2 Changes in tumor PDH activity induced by alcohol derived acetaldehyde as a potent carcinogen.

High alcohol intake is strongly linked with increased cancer incidence in the upper respiratory tract, liver and probably colon, rectum and breast (Franceschi et al., 1994; Lof and Weiderpass, 2009; Huxley et al., 2009). Some cancer cells exhibit increased expression of alcohol dehydrogenase (ADH) and changes in aldehyde dehydrogenase (ALDH) isoforms to those with reduced affinity for the substrate acetaldehyde (Jelski and Szmitkowski, 2008). This has resulted in the proposal that at least liver, esophagous, stomach and colorectum cancers have a greater capability for ethanol oxidation to produce acetaldehyde, but less ability to remove acetaldehyde than normal tissues (Jelski and Szmitkowski, 2008). ALDH2, the mitochondrial isozyme, is primarily responsible for the oxidation of acetaldehyde, because the ALDH1 isoform is cytosolic with a 10-fold higher *K_m* value. Consequently, cancer cells expressing low amounts of ALDH protein and ALDH activity (Jelski and Szmitkowski, 2008), are less able to oxidise acetaldehyde to

acetate resulting in increased levels of acetaldehyde, particularly in response to alcohol intake when alcohol dehydrogenases (ADH) converts alcohol into carcinogenic acetaldehyde.

ROS in the form of $O_2\cdot^-$ may also be carcinogenic, since it directly inhibits the mitochondrial ALDH2 required to inactivate carcinogenic acetaldehyde by converting it into acetate (Deitrich et al., 2007). In support of the significant role played by acetaldehyde in cancer, alcohol-consumers with a defective ALDH2 allele who consume alcohol are highly predisposed to squamous cell carcinomas of the upper digestive tract (Deitrich et al., 2007).

Acetaldehyde, as a highly reactive compound not found in high levels during normal cell metabolism, induces radical changes in cancer cells by reacting with (a) primary amines on cellular proteins; (b) free sulphhydryl groups; and (c) DNA to form adducts (Lieber, 1988a,b; Baggetto, 1992; Niemelä, 1999; Brooks and Theruvathu, 2005). In addition, acetaldehyde in some cancer cells is produced as an active by-product of PDH (Baggetto and Lehninger, 1987; Baggetto and Testa-Parussini, 1990). PDH is associated with the inner mitochondrial membrane (Margineantu et al., 2002) and in normal cells PDH decarboxylates pyruvate, transforming it into acetyl-CoA which then feeds into the Krebs's cycle to produce citrate in the mitochondria. Hence, PDH links the cytosolic glycolytic pathway to the mitochondrial Krebs's cycle.

By contrast, in some cancer cells, PDH can produce and/or use acetaldehyde, to turn itself into a non-oxidative decarboxylase, converting more pyruvate to acetoin and finally to acetaldehyde. It should be noted that acetaldehyde was not detected in mitochondria from Ehrlich ascites tumors (Baggetto and Lehninger, 1987), and this probably results from its rapid reaction within PDH, at the level of the first enzyme in the PDH complex [PDH-E1 or pyruvate decarboxylase]. PDH-E1 performs the first two reactions within the pyruvate dehydrogenase complex: a decarboxylation of pyruvate and a reductive acetylation of lipoic acid. Lipoic acid is covalently bound to dihydrolipoamide acetyltransferase (E2), the second catalytic component enzyme of the PDH complex.

In cancer cells, acetaldehyde undergoes rapid reaction with decarboxylated pyruvate (as a hydroxyethyl-thiamine-pyrophosphate-[HE-TPP] enzyme complex) to form acetoin, a neutral compound that like acetaldehyde, is not normally present in mammalian cells (Fig. 1) (reviewed in Baggetto, 1992). PDH-E1 can also use acetaldehyde directly, as an alternative substrate to pyruvate, when it condenses two molecules of acetaldehyde to form acetoin (Alkonyi et al., 1976). Hence, the production of acetoin as a neutral by-product reducing any build up of reactive and toxic acetaldehyde in cancer cells acts to detoxify it in the tumor mitochondria (Baggetto and Testa-Parussini, 1990). The reaction from pyruvate to acetoin occurs at very high rates (from 35 $\mu\text{mol CO}_2/\text{min/g}$ protein) when analysed in isolated mitochondria from some tumors, regardless of the presence or absence of oxygen, indicating non-oxidative decarboxylation (Baggetto and Lehninger, 1987). By contrast, in normal rat liver, the PDH reaction is tightly controlled by oxygen levels to which it is coupled so that the respiratory quotient of pyruvate utilization is close to 1.0 for both state 3 (ADP-stimulated, ATP synthesis state) and state 4 (basal, non-phosphorylating state) respiration, in line with 1:1 oxidative decarboxylation of pyruvate to yield acetyl-CoA.

2.3 Inhibition of PDH activity by Pyruvate Dehydrogenase Kinase (PDK) in cancer cells.

On the basis of the markedly reduced activity of PDH in Ehrlich cancer cells, it was proposed that a different isoform of the PDH enzyme must exist (Baggetto and Lehninger, 1987), reflecting similar changes to many other glycolytic enzymes found in cancer cells (reviewed in Marín-Hernández et al., 2009). However, this is not the case. Only one X-linked gene exists that encodes PDHA1, the subunit in the PDH complex that acts as the pyruvate decarboxylase. Mutations in the human gene results in potentially fatal lactic acidosis (reviewed in Lissens et al., 2000), in agreement with the reduced PDH activity in some cancer cells increasing lactate production.

The greatly reduced activity of the PDH complex in some cancer cells was subsequently found to result from the binding and phosphorylation of PDH subunits by a family of regulatory enzymes known as the PDH kinases (PDKs) (reviewed in Roche and Hiromasa, 2007). The PDK family includes four known members (PDK1-4), being PDK1 the most prominent activity in cancer cells. PDKs bind to the outer (L1) or inner lipoyl domain (L2) of PDH-E2 or lipoyl domain of PDH-E3. PDK1 preferentially binds tightly to the L2 free lipoyl domain thereby directly increasing kinase activity, which then modulates PDH-E1 by phosphorylating three serine residues in the alpha subunit of PDH-E1 (reviewed in Roche and Hiromasa, 2007). The action of phosphorylation, even of a single serine residue in PDH-E1 significantly reduces the pyruvate decarboxylase activity (Patel and Korotchkina, 2001). It is interesting to note that when Ser264 (site 1 of PDH-E1) is replaced by glutamine, the activity with pyruvate is only reduced ~40%, whereas the activity with the substrate analog, α -ketobutyrate, is increased by about 5-fold. Hence, it is possible that modification by PDK could alter the PDH specificity by promoting the acetaldehyde to acetoin enzymatic reaction (see section 2.2 above for details). The phosphate group in the PDH-E1 active site affects E1 interaction with the negatively charged residues of the lipoyl domain because of its size and anionic nature, affecting reductive acetylation in the second reaction of the PDH complex (Liu et al., 2001).

Lipoic acid is a fatty acid with a disulphide-containing heterocyclic structure with antioxidant properties. It is also conjugated to proteins as an important cofactor of PDH and 2-oxoglutarate dehydrogenase (2-OGDH) in the Krebs's cycle. Treatment of some cancer cells with lipoic acid promotes ROS production and induces apoptosis (Simbula et al., 2007), because lipoic acid is an inhibitor of PDK's, which increases PDH complex activity (Korotchkina et al., 2004) and restores the flow of pyruvate into the Krebs's cycle to promote OxPhos. Despite the established function of lipoic acid as an antioxidant in normal cells, it can act as a pro-oxidant when added to cancer cells, inducing elevated ROS, p53 and caspase activation leading to apoptosis (Moungjaroen et al., 2006; Simbula et al., 2007). Although still in the early phase, lipoic

acid has been used in preclinical (murine leukemias) and clinical cancer trials (pancreatic cancer and a range of solid tumors) purportedly on the basis of its antioxidant properties. Thus, lipoic acid has enhanced clinical outcomes with no associated toxicity when combined with chemotherapeutic agents like doxorubicin or IL-2 and methoxyprogesterone acetate (reviewed in Novotny et al., 2008).

It is interesting that a second inhibitor of PDK's, dichloroacetic acid (DCA) has also been shown to switch cancer cell metabolism from anaerobic glycolysis to increase mitochondrial respiration and ROS production, promoting apoptosis and inhibiting cancer growth (Bonnet et al., 2007). Thus, inhibiting PDK's rapidly increases the flux of pyruvate into tumor mitochondria, inducing excessive ROS production and activates cancer cell apoptosis. Not surprisingly, approaches using such metabolic targeting therapies are rapidly gathering momentum as promising novel treatments for cancer (reviewed in Michelakis et al., 2008; Ralph and Neuzil, 2009). Unfortunately, DCA is not specific for tumor PDH and similar doses inhibiting the tumor enzyme also decrease other metabolic pathways causing toxicity to the normal tissues as well (Cai et al., 2007).

2.4 HIF, hypoxia, pseudohypoxia, succinate and targeting complex II: The importance of targeting mitochondrial bioenergetic pathways in tumor cells.

Many metabolic changes in cancer cells are mediated by the activation of hypoxia inducible factor (HIF), which increases when oxygen levels diminish leading to hypoxia in tumors (reviewed in Marín-Hernández et al., 2009). Under aerobiosis, HIF α prolyl hydroxylases (PHD's) maintain HIF α subunit turnover by promoting its degradation, whereas under hypoxia PHD's become inactivated. The major modifications induced by HIF include increased PDK and LDH activity rerouting pyruvate metabolism away from the mitochondria, increasing rates of glycolysis as well as many other significant metabolic changes (reviewed in Marín-Hernández et al., 2009). In addition, in cancer cells where succinate dehydrogenase (SDH) or fumarate

hydratase (FH), as components of the Krebs's cycle, become mutated to lose their activity, levels of succinate or fumarate increase and the cancer cells undergo a major event in malignant transformation involving HIF activation, even under normoxic conditions. This situation is known as "pseudohypoxia" and in the SDH- or FH-defective cells, HIF activation is due to HIF1alpha protein subunit stabilization because excessive build up of succinate or fumarate inhibits the PHD enzyme that regulates HIFalpha subunit turnover (reviewed in Boulahbel et al., 2009).

HIF activation by hypoxia or pseudohypoxia is a common trait of cancer cells and unfortunately has provided much support for the misleading concept of anaerobic glycolysis driving cancer cell metabolism - a notion that has become integrally linked with the "Warburg's Hypothesis" used to describe tumor cell metabolism. The enzyme SDH comprises the matrix facing subunit, SDHA, associated with the remaining subunits (SDH B, C and D) of complex II (CII) which are embedded in the mitochondrial inner membrane, forming part of the respiratory chain. According to the prevailing dogma, respiratory chain function and OxPhos should be deficient in tumor cells. However, much of this review indicates that the opposite situation is more likely in that mitochondrial bioenergetic pathways remain essential to the growth and survival of tumor cells, which makes these pathways an excellent choice for targeting to kill tumors. Additional support for this proposal derives from our studies using the novel mitocans, Vitamin E analogues, which has shown that these compounds are excellent anticancer agents that act by targeting the mitochondria in tumor cells (reviewed in Neuzil et al., 2007). Alpha-tocopheryl succinate (α -TOS) is a selective inducer of apoptosis in cancer cells, involving the rapid accumulation of ROS. The molecular target of α -TOS was identified and α -TOS shown to preferentially inhibit succinate dehydrogenase (SDH) activity of complex II (CII) by interacting with the proximal and distal ubiquinone (UbQ)-binding site (Q_P and Q_D , respectively) (Dong et al., 2008). It was proposed that α -TOS displaced UbQ in CII causing electrons generated by SDH, and retained in the prosthetic group $FADH_2$, to recombine with molecular oxygen to yield ROS; hence suppression of tumor growth *in vivo* by this drug required a fully functional CII, establishing

CII as a novel target for cancer therapy and arrested the growth of tumors in pre-clinical models (Dong et al., 2009; reviewed in Ralph and Neuzil, 2009).

In Ehrlich tumor cell mitochondria, succinate oxidation by CII of the respiratory chain was inhibited by high levels of acetoin (1mM), which indicated an additional mechanism for reducing mitochondrial respiratory function in tumor cells in the presence of glucose (Baggetto and Testa-Parussini, 1990) whereas normal rat liver mitochondria were unaffected by 1mM acetoin. The PDH complex from Ehrlich ascites tumor cells was also competitively inhibited by acetoin, whereas that from normal rat liver was not, again indicating that significant differences exist between normal and cancer cells in inhibitor affinities and state of the PDH and SDH/CII enzyme complexes (Baggetto and Lehninger, 1987; Baggetto and Testa-Parussini, 1990). A prediction from these results is that acetoin accumulation in cancer cells will inhibit the SDH/complex II causing increased succinate levels which will then inhibit PHD activity and lead to the activation of HIF, promoting the bioenergetic transformation of malignant cells.

Acetoin in turn, is rapidly converted to citrate, albeit by an undefined reaction mechanism, thereby enhancing the rate of citrate production in cancer cells (Baggetto, 1992). ¹⁴C acetoin was first found to be utilized by normal liver slices for the biosynthesis of either cholesterol or fatty acids, albeit poorly compared to the readily useable substrate, acetoacetate (Brady et al., 1951). However, purified mitochondria from Ehrlich ascites tumor cells or normal rat liver were later shown to efficiently use acetoin to produce citrate at similar rates (Baggetto and Testa-Parussini, 1990). The citrate produced is rapidly extruded from tumor mitochondria, feeding into cytosolic fatty acid synthesis (reviewed in Mycielska et al., 2009). The end result of this alternative routing of pyruvate/acetoin/citrate exiting the mitochondria is to create a truncation point in the Krebs' cycle in tumor cells (Fig. 1).

The net outcomes from HIF activation and acetoin production are that mitochondrial oxygen consumption and ATP production are reduced and cancer cells mainly survive on glycolysis, if sufficient glucose is available. However, a caveat to this interpretation is that cancer

cells can switch their metabolism to survive on fatty acids, ketone bodies or by amino acid oxidation (covered in sections 4, 5, and 8 below).

3. Alternative ATP synthesis pathways in cancer cells.

The most characterized change in cancer cell energy metabolism is their increased glycolytic capacity even in the presence of a high O₂ concentration (Warburg, 1956; Pedersen, 1978; Mazurek et al, 1997; Rodríguez-Enríquez et al., 2000; Gatenby and Gillies, 2004; Griguer et al, 2005; Marín-Hernández et al., 2006). Warburg (1956) originally proposed that the driving force for enhanced glycolysis in tumor cells was the energy deficiency caused by an irreversible damage of the mitochondrial function. Since then, it has become widely assumed that ATP supply is mainly or only provided by glycolysis in **all** types of tumors, without thorough experimental demonstration and careful evaluation. Tumor cell lines certainly exist that exhibit decreased mitochondrial function, but this is not a general phenomenon applicable to all tumor cells. Therefore, because of the genetic heterogeneity of tumor cells, it is important that the capacity for OxPhos be experimentally evaluated for each particular tumor and an assessment made whether or not their enhanced glycolysis is indeed accompanied by significantly depressed mitochondrial function.

Most cancer cell lines exhibit enhanced rates of glycolysis (reviewed in Moreno-Sánchez et al., 2007; 2009; Marín Hernández et al., 2009) because they over-express all the enzymes and transporters of the pathway, particularly GLUT, HK and PFK-1 (Marín-Hernández et al., 2006; Rodríguez-Enríquez et al., 2008; 2009). However, much less attention has been given to the enhanced mobilization and oxidation of fatty acids (FA) observed during cancer cachexia (Russell and Tisdale, 2002) and its role in the production of tumor cell ATP. Glutamine is also consumed at high rates by tumor cells and again the role of glutamine oxidation in supplying cellular energy has not been fully explored.

If glycolysis is the main ATP supply pathway in cancer, then the main carbon source consumed by tumors should be glucose. Indeed, nutrients such as glucose contained in the host arterial blood are consumed by fast-growing solid tumors and xenografts at about 10-50% of the blood supply rate. However, in addition to glucose, several other carbon sources including lactate, amino acids (mainly glutamine), fatty acids and ketone bodies are not only actively utilized for tumor cell growth (in macromolecule biosynthesis), but also for energy requirements (*i.e.*, oxidative metabolism; see Table 1) (Rodríguez-Enríquez et al., 2000; 2006; reviewed in Sauer et al., 2006). Therefore, although glycolysis might be a major pathway for ATP supply in solid tumors, mitochondrial oxidative pathways and OxPhos may still hold significant roles.

Many cancer cell studies have relied on analysis of mRNA expression or protein levels in order to reach conclusions about the functional relationships of bioenergetics operating inside tumor cells. Thus, the direct determination of transporter or enzyme activity or content has seldom been accomplished. It is usually assumed that transcriptional changes correspond to increased activity and pathway flux. Lamentably, the required biochemical (metabolic, kinetic) experimentation is considered “old-fashioned” or “technically difficult” and, only a few studies have reported transporter function, enzyme activities and flux rates.

Recent research analysing the regulation of *Saccharomyces* metabolism and gene expression has firmly established that transcription and post-transcriptional processes make a negligible contribution to the control of enzyme activity and pathway flux (Rossell et al., 2005; Postmus et al., 2008). Most of the control resides in post-translational metabolic (kinetic) mechanisms such as allosteric and covalent (phosphorylation/dephosphorylation) modulation. Moreover, it is also widely observed that a variation in mRNA content does not always lead to associated changes in enzyme (or transporter) content or activity. Furthermore, it is important to note that differences in enzyme activity do not automatically bring about a change in pathway flux or metabolite concentration, unless the affected enzyme has a major role in flux- and concentration-control (for further detail, refer to Moreno-Sánchez et al., 2008).

4. Fatty acid metabolism and mitochondrial function in cancer cells.

Increasingly the evidence is mounting that implicates an important role for dietary fat-gene interactions in the initiation and progression of human cancer. Thus, the development of an appropriate diet that provides effective cancer prevention is becoming an important and novel area of research. Some dietary products appear to up-regulate tumor growth whereas others down-regulate growth. Common knowledge would suggest that diets low in fat and red meat, but rich in fish, vegetables, fruits, herbs and spices decrease risk of cancer development. Therefore, high fat diets (HFD's) should be avoided as they may stimulate tumor promotion. In oncogenic K-ras activated mice, a HFD certainly increased energy metabolism through increased expression of mitochondrial β -oxidation genes (CPT-I, ACC, PPAR γ) that contribute to greater glucose tolerance (Khasawneh et al., 2009). However, HFD leads to obesity which in turn increases the risk for gastrointestinal cancers. Epidemiological studies also suggest a strong correlation between obesity, insulin resistance and pancreatic cancer, while a correlation between dietary fat and prostate (Fleshner et al., 2004) or metastatic colon cancer (VanSaun et al., 2009) has been described. It is thought that intake of low fat vegetarian food has a protective role against prostate cancer incidence (Chen et al., 2005).

Cancer cells can also show significantly increased levels and activity of the enzymes involved in metabolising fatty acids, both for the synthesis and degradation of these energy sources. In contrast, in Ehrlich hepatoma, rhabdomyosarcoma and Becker transplantable hepatocellular carcinoma H-252, the oxidation of fatty acids was extremely reduced compared to normal liver (Weinhouse et al., 1953; Cederbaum and Rubin, 1976). The link between the synthesis and degradation of fatty acids in cancer cells is not yet clearly defined or why both systems operate simultaneously at higher levels in some cancer cells but not others. However, it is likely that both metabolic pathways are required to provide essential factors involved in cancer cell growth and survival, such as the supply of cholesterol and phospholipids for membranes and

cofactors such as lipoic acid for enzyme function. The reasons for maintaining catabolism and anabolism of fatty acids at high levels in some cancer cells are the subject of the next sections, particularly as they relate to mitochondria and the potential for targeting these processes to develop improved and specific anticancer drugs for those tumors with elevated fatty acid metabolism.

4.1 Fatty acid synthesis in cancer, the mitochondrial connection and its targeting as a therapy.

Two enzymatic complexes exist inside mammalian cells that function to produce different fatty acid species. The predominant form, fatty acid synthase I [FAS I, also known as FASN] is localised in the cytosol and is ancestrally related to the polyketide synthases of lower eukaryotes (reviewed in Kuhajda, 2006; Menendez and Lupu, 2007). Cancer cells commonly express increased levels of FAS I (reviewed in Little and Kridel, 2008), although in patients carrying p53 mutations, the FAS activity of colorectal carcinoma was lower than in normal tissue (Notarnicola et al., 2006). This large multi-enzyme complex catalyses all steps of fatty acid synthesis from acetyl-CoA, malonyl-CoA and NADPH, producing longer chain fatty acids. Although the cytosolic location of FAS I might indicate a lack of connection with mitochondrial bioenergetics, this is not the case because citrate, derived from tumor cell mitochondria (see above) acts as a major carbon fuel for fatty acid, triacylglycerol or cholesterol synthesis in the cytosol (Fig. 2, glutaminolysis pathway). First, however, citrate must be exported from the mitochondria to the cytosol via the tricarboxylate transporter. This transporter exchanges dibasic forms of tricarboxylic acids (*e.g.* citrate, isocitrate and cis-aconitate) for other tricarboxylates, dicarboxylates or phosphoenolpyruvate. Thus, citrate is transported from the mitochondrial matrix to the intermembrane space to then diffuse across the outer mitochondrial membrane into the cytosol. The tricarboxylate transporter may play an important role in the aberrant bioenergetics of some tumors (Kaplan et al., 1982), particularly prostate cancer (reviewed in Mycielska et al.,

2009). To date, no specific studies have been reported that examine the importance of this protein in cancer, using either RNAi or inhibitory drugs.

In the cytosol, ATP citrate lyase (ACL) reconverts citrate to acetyl-CoA and acetyl-CoA carboxylase (ACAC) also requires ATP to carboxylate acetyl-CoA, producing malonylCoA (Fig. 4). Chemical inhibition of ACAC with the macrocyclic polyketide, soraphen A, selectively induced growth arrest and cytotoxicity of cancer cells (Beckers et al., 2007). Soraphen A bound to the active site dimerization domain of ACAC, mimicking the blocking of ACAC activity by AMPK-mediated phosphorylation of ACAC (Cho et al., 2009). No clinical or preclinical studies with soraphen A could be found.

The next step in fatty acid synthesis involves FAS I condensing malonyl-CoA with acetyl-CoA to produce palmitate and other saturated long-chain FA (LCFA's). The structure of FAS I involves a large multienzyme system with a complex architecture of alternating linkers between five enzymatic domains. Substrate shuttling is facilitated by flexible tethering to the acyl carrier protein (ACP) with limited contact in this multienzyme between the condensing and modifying portions, which are mainly connected by linkers rather than direct interacting with each other (Maier et al., 2008).

The other fatty acid synthase complex, FAS II is located in the mitochondrial matrix (reviewed in Schneider et al., 1997; White et al., 2005; Hiltunen et al., 2009) and is related to the prokaryotic system for type II fatty acid synthesis which is the principal means for producing membrane phospholipid acyl chains in bacteria and plants. The FAS II reaction sequence occurs via a series of individual enzymes and similarly to FAS I, the pathway intermediates are shuttled between the enzymes as thioesters linked to an acyl carrier protein (ACP). The major function of the FAS II system relevant to cancer is that it contains the enzymes required for the extension of a 2-carbon precursor by the serial addition of malonyl moieties to produce saturated acyl-ACP thioesters containing up to 14 carbon atoms (reviewed in Hiltunen et al., 2009). A major product of FAS II is octanoyl-ACP required for mitochondrial protein lipoylation. As described above in

section 2.1.2, lipoic acid is an essential cofactor required for many important enzyme complexes, including the mitochondrial complexes PDH, 2-OGDH, and the glycine cleavage system.

ACP holds several important functions in mammalian mitochondria (Feng et al., 2009). First, as a key component of the mitochondrial fatty acid biosynthetic pathway, ACP plays an essential role providing octanoyl-ACP precursor required for protein lipoylation linking fatty acid synthesis to mitochondrial respiration via PDH and 2-OGDH. Second, as a subunit of complex I, ACP is required for the efficient functioning of the electron transport chain, low production of ROS and maintenance of normal mitochondrial membrane potential. The lipoic acid moiety is attached to the E2 subunit of PDH and is required for the conversion of pyruvate to acetyl-CoA, which feeds into the FAS pathway. This connection may provide a regulatory feedback between FAS and mitochondrial metabolism (Witkowski et al., 2007). Hiltunen et al. (2009) proposed that when the pyruvate supply is low, less acetyl-CoA would be available for the FAS pathway, resulting in lower levels of lipoic acid and hence, reduced PDH activity.

A third connection also exists. In vertebrates, the mitochondrial 3-hydroxyacyl-acyl carrier protein dehydratase and the RPP14 subunit of RNase P are encoded by the same bicistronic transcript in an evolutionarily conserved arrangement. A positive feedback loop exists in the yeast mitochondrial RNase P maturation process, in which processing of the RNase P RNA precursor, RPM1-tRNAPro, requires the action of the RNase P enzyme itself (reviewed in Hiltunen et al., 2009). The data have shown these two regulatory loops are linked; disruption of the FAS pathway results in lower levels of processed RPM1-tRNAPro precursor RNA and all other mitochondrial tRNA's. Although not substantiated experimentally, this feedback loop may have a direct effect on the synthesis of mitochondrial encoded components of the respiratory chain, with ACP as part of mitochondrial complex I providing a further link. As a result, diminished respiratory chain function could affect the maintenance of the respiratory chain and normal mitochondrial membrane potential (Feng et al., 2009).

Inhibitors of FAS based on the antifungal antibiotic cerulenin have been tested on cancer cell lines and human tumor xenografts *in vivo*. Cerulenin itself is not suitable as it contains a reactive epoxy group and has a broad spectrum of action as an inhibitor of fatty acid and steroid biosynthesis, but it also stimulates fatty acid metabolism by β -oxidation (reviewed in Menendez and Lupu, 2007). Cerulenin binds β -keto-acyl-ACP synthase, one of the enzymatic components of the FAS complex, preventing binding of malonyl-CoA. In addition, the drug promotes mitochondrial fatty acid oxidation by activating carnitoyl palmitate transferase-1 (CPT1) preventing its inhibition by malonyl-CoA. The action of cerulenin involves covalent thioacylation thereby leading to permanent changes in the activity of target enzymes. Synthetic analogues of cerulenin acting as more stable or specific inhibitors of FAS, such as C75 and C93, have been developed (Kuhajda, 2006; Orita et al., 2007). C75 was the first synthetic FAS I inhibitor that was more stable than cerulenin and showed significant anticancer activity as a proapoptotic drug against a range of human cancer cell lines as well as *in vivo* xenografted preclinical tumor models (reviewed in Kuhajda, 2006). However, C75, like cerulenin, shows dose-limiting toxicities by inducing substantial weight loss in animals by stimulating CPT1 activity, and it promotes anorexia associated with increased mitochondrial fatty acid oxidation, affecting normal tissues. It is not clear how C75 promotes CPT1 activity as it does not compete for the malonyl-CoA binding inhibitory site on CPT1. Instead, it has a distinct and unknown mechanism possibly by modifying the relationship of CPT1 with the mitochondrial membrane (Yang et al., 2005).

Previous studies (Fraser et al., 2001) showed that CPT1 is highly enriched at the contact sites between the mitochondrial outer (MOM) and inner (MIM) membranes and its activity is regulated by the membrane environment, affecting the long chain acyl-CoA binding site and kinetics of CPT1. Gamma-linoleic acid (GLA, an essential omega-6 PUFA) inhibits Walker 256 tumor growth *in vivo*, reducing the number of CPT1 mitochondrial membrane contact sites, inhibiting CPT1 and hexokinase association with the MOM, as well as inhibiting β -oxidation thereby increasing the triacylglycerol content of tumors (Colquhoun, 2002).

Recently, a second drug C93 was described as a more specific inhibitor of FAS I without the action on CPT1, fatty acid oxidation or anorexia. C93 given orally or intraperitoneally at 100mg/kg/day significantly inhibited the growth of xenograft transplanted human lung cancers in nude mice without affecting fatty acid oxidation or animal weight and it was encouragingly non-toxic (Orita et al., 2007). Similar results were reported with the human ovarian cancer line, SKOV3 and C93 was also non-toxic in pharmacological studies with rats and dogs (El Meskini et al., 2008). To date, no clinical trials in cancer patients have been reported, but they are eagerly awaited.

Kuhajda's group has proposed that FAS inhibition, but not enhanced fatty acid oxidation, is required for the selective anticancer effects of drugs like C93. Other potent and selective inhibitors of FAS have been discovered. Natural products inhibiting FAS I include flavanoids, some with μM IC₅₀ values (Li and Tian, 2004) and (-)-epigallocatechin-3-gallate (EGCG), the green tea polyphenol, which induces apoptosis in breast cancer cells without affecting CPT1 or animal body weight (Puig et al., 2008). More studies are required to assess the clinical efficacy of such compounds in cancer trials (reviewed in Thomasset et al., 2007).

Another FAS inhibitor with an apparent $K_i \sim 100\text{nM}$, affecting the last enzymatic thioesterase step is tetrahydrolipstatin (Orlistat, Xenical), containing a reactive β -lactone group. Orlistat is an FDA-USA approved oral drug treatment for preventing dietary lipid uptake by inhibiting lipase action; in drug screening, it inhibited growth of a range of different cancer cell lines by inducing apoptosis, without affecting normal cells. Orlistat's inhibitory effects were reversed by addition of palmitate, the end product of FAS. Closer analysis showed that Orlistat inhibited several human prostate cancer cell lines in culture [1-25 μM] and i.v. injections into nude mice produced peak blood levels $\sim 10\mu\text{M}$ and inhibited growth of the PC-3 prostate cancer line as tumor xenografts (Kridel et al., 2004). Crystal structural analysis revealed that Orlistat bound tightly into the thioesterase active site as an unusually stable acyl-enzyme intermediate inhibiting its function (Pemble et al., 2007). The C16 hydrocarbon backbone of Orlistat mimicked palmitate,

binding along a hydrophobic channel that would help to explain why high palmitate levels prevented the drug's inhibitory action on cancer cells and may be a limiting factor in its use. Orlistat is also not very specific for the tumor enzyme because at low doses (nM range) this drug also affects rat liver triacylglycerol lipase, N-acylphosphatidyl-ethanolamine-selective phospholipase D, fatty acid amide hydrolase and human recombinant cannabinoid CB1 and CB2 receptors (Bissogno et al., 2006).

The mechanism whereby FAS I inhibition specifically induces cancer cell death by apoptosis is not clearly defined. However, one possibility is that blocking the fatty acid metabolic pathways increases malonyl-CoA levels, and consequently, the reversible reaction of malonyl-CoA transferase may return malonyl-CoA back to toxic malonate plus acetyl-CoA. Kuhajda's group showed by using the selective inhibitor 5-(tetradecyloxy)-2-furoic acid (TOFA) to block ACAC (acetyl-CoA carboxylase) that the anticancer activity of C75 against breast cancer was mediated by the associated toxic fluxes due to build up of malonyl-CoA (Pizer et al., 2000). Further studies showed that the anticancer effect of cerulinin could be mimicked by blocking both, fatty acid oxidation by inhibiting CPT1 with the selective inhibitor etomoxir, and blocking fatty acid synthesis and FAS I (via ACAC) with TOFA (Thupari et al., 2001). TOFA mediated FAS inhibition alone is cytotoxic for a range of human cancer cell lines, inducing apoptosis (Wang et al., 2009). Alone, etomoxir mediated CPT1 inhibition also induces ROS and impairs mitochondrial energy metabolism in HepG2 liver cancer cells (Merrill et al., 2002). In addition, inhibiting malonyl-CoA decarboxylase, that would otherwise remove malonyl-CoA to form acetyl-CoA and CO₂, is selectively cytotoxic for cancer cells (Zhou et al., 2009). All of these pieces of evidence point to a major role for the enzymes that control malonyl-CoA concentration as an important metabolic switch acting as a substrate required for FAS as well as an inhibitor of fatty acid oxidation, so that any build up in levels of malonyl-CoA precipitates an energy crisis and is toxic for cancer cells.

The question arises how the build up in malonyl-CoA kills cancer cells. The most straightforward possibility is that increased levels of malonyl-CoA reverse the reaction of malonyl-CoA transferase and raises malonate, which is a recognised strong competitive inhibitor of both the succinate (dicarboxylate) transporter and complex II/succinate dehydrogenase (SDH) activity (Pardee and Potter, 1949; Kotliar and Vinogradov, 1984), leading to mitochondrial ROS production and cancer cell death by apoptosis (Fernandez-Gomez et al., 2005; Gomez-Lazaro et al., 2007). In addition, malonyl-CoA and similar metabolic intermediates, including long-chain acyl-CoA esters could block or compete for the mitochondrial dicarboxylate transporter (Ventura et al., 2005), preventing the flow of citrate or succinate, adding to the oxidative stress.

4.2 Fatty acid degradation by β -oxidation.

Use of fatty acids as a metabolic source for mitochondrial fatty acid oxidation provides cancer cells with another alternative energy supply when triacylglycerides are available or glucose is limiting. As described in the previous section, the evidence indicates that both fatty acid synthesis and degradation occur simultaneously at high levels in some cancer cells in a seemingly futile cycle (also see Menendez et al., 2005). In this regard, it is surprising that changes in the expression of the mitochondrial β -oxidation transporters and enzymes in cancer cells to date have been a relatively understudied area (however, see PPAR section below for indirect evidence of changes in cancer cell fatty acid degradation). One study used NMR on murine mammary carcinoma/sarcoma EMT6 tumor cell spheroids to obtain evidence of fatty acid degradation in cancer cells. ^{13}C -propionate was used to monitor metabolic changes in the spheroids (with glucose and glutamine present) and the radiolabel was incorporated into succinate, malate and glutamate, supporting direct fatty acid degradation (Wehrle et al., 2000).

After cytosolic modification by acyl-CoA synthetases, FA's are oxidized in both mitochondria (Fig. 2) and peroxisomes by β -oxidation and in the endoplasmic reticulum by ω -

oxidation (reviewed in Yu et al., 2003). Lactate, glutamine and ketone bodies (KB's) are also fully oxidized in the mitochondrial matrix with the participation of the Krebs's cycle (Fig. 2).

Mitochondrial β -oxidation primarily transforms short-chain (<C8), medium-chain (C8–C12), and long-chain (C12–C20) saturated and unsaturated fatty acids into acetyl-CoA, thereby feeding either the Krebs's cycle or ketogenesis (reviewed in Bartlett and Eaton, 2004); KB's normally serve as oxidizable substrates for extrahepatic tissues (Fig. 2). The outer membrane carnitine-acylCoA or carnitine-palmitoyl transferase (CPT-I) generates acyl-carnitine derivatives, which are transported into the mitochondria by a specific inner membrane transporter in exchange for internal free carnitine. The inner membrane CPT-II removes carnitine from the internalized acyl-carnitine derivatives and introduces CoA. Next, the acyl-CoA ester is dehydrogenated by acyl-CoA dehydrogenases specific for very-long-chain (VLCAD), long-chain (LCAD), medium chain (MCAD), and short-chain (SCAD) fatty acids.

The next three steps consisting of 2-enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase are catalyzed by the mitochondrial trifunctional protein (MTP). MTP is a heterotrimeric protein of four α -subunits and four β -subunits and mainly catalyzes long-chain fatty acid oxidation. Reddy and Rao (2006) proposed that mitochondrial β -oxidation is regulated by CPT-I, depending on carnitine (as a limiting substrate) and malonyl-CoA concentrations (as a potent competitive CPT1 inhibitor).

Peroxisomal β -oxidation metabolizes less abundant, but more complex, toxic and biologically active FA's such as very-long-chain fatty acids (\geq C20), 2-methyl-branched fatty acids, and long-chain dicarboxylic acids. Thus, peroxisomal β -oxidation is required to process the more complex branched chain fatty acids (BCFA's) and the role for mitochondrial β -oxidation here is less certain. BCFA's are important components of the human diet and can be degraded by α -, β - or ω -oxidation.

Dietary BCFA's (especially phytanic acid) have attracted much attention in recent years, due to their strong link with prostate, breast, colon and other cancers, as well as their role in

neurological disease. The enzyme α -methylacyl CoA racemase (AMACR) located in peroxisomes and mitochondria, catalyses the racemisation of α -methyl and carboxylic branched chain acyl-CoA thioesters, preparing them for metabolism by peroxisomes and mitochondria. Interestingly, AMACR is a tumor marker and is overexpressed by a large range of cancers (reviewed in Lloyd et al., 2008). Thus, AMACR plays a central role in regulating the metabolic pathways for BCFA by acting as the gatekeeper controlling entry into the peroxisomal and mitochondrial β -oxidation pathways.

AMACR functions in the chiral inversion of a range of 2-methyl acids (as their CoA esters), and may have an important role in carcinogenesis (reviewed in Lloyd et al., 2008). Preliminary data have shown that targeting AMACR with inhibitors [K_i values in the μM range] prevents growth of cancer cell lines with potency corresponding to the cellular level of AMACR protein (Carnell et al., 2007). Two theories exist for the role of BCFA and AMACR in cancer (reviewed in Lloyd et al., 2008). The first is that BCFA can directly promote ROS production, probably by affecting respiratory chain complexes, inducing oxidative stress and DNA damage. The second is that by acting as high affinity binding ligands, they alter the function of receptors such as the PPAR's and RXR's known to be involved in regulating expression of fat metabolising enzymes. Given these or other possibilities, more research into BCFA function is required.

The beneficial roles of dietary fish oil in lowering serum triacylglycerol levels in animals and humans have been attributed in part to the high content of two n-3 polyunsaturated very long-chain FA, eicosapentaenoic acid (EPA), and docosahexanoic acid (DHA) (Fig. 3). As a family of polyunsaturated fatty acids (PUFA's), they are popularly referred to as omega-3 fatty acids, sharing a final carbon-carbon double bond in the n-3 position, the third bond from the methyl end of the fatty acid. Recent studies show that EPA induces mitochondrial β -oxidation in hepatocytes by acting as a PPAR agonist, which contributes to their systemic lipid-lowering properties. EPA increases oxidation of endogenous FA but does not inhibit lipogenesis. In contrast, oleic acid (a monounsaturated omega-9 fatty acid) or clofibrate does not affect FA oxidation or lipogenesis

while octanoic acid suppresses both the oxidation of endogenous FA and lipogenesis. Increased β -oxidation by EPA was associated with increased CPT-1 activity but without changes in its mRNA or in protein expression. EPA treatment increased the percentage of this FA in mitochondrial membrane lipids and it was proposed that EPA directly increased the activity of CPT-1 and β -oxidation in adipocytes by altering the structure or dynamics of CPT-1 interaction with the mitochondrial membranes (Guo et al., 2005). This conclusion is consistent with the effects of C75 and cerulenin in promoting CPT1 activity described above as acting by modulating CPT1 membrane association. Other studies have shown that oxidation of the PUFAs might also be important for their functional activation (Itoh and Yamamoto, 2008).

4.3 Uncoupling protein 2

Cancer cells obtain additional selective survival advantages from their increased rates of fatty acid (FA) β -oxidation-derived energy supply. In normal cells with sustained glucose levels and the presence of insulin, regulatory links exist that set the rate of glycolysis *versus* the rate of FA β oxidation, to allow for substrate switching depending on lipid availability and this has been termed the Glucose Fatty Acid Cycle or “Randle cycle” (Randle et al., 1963). Thus, in normal rat muscle supplied with FA’s or ketone bodies (KB’s), glucose uptake, glycolysis and glucose oxidation become impaired (Randle, 1963; 1964) because increased acetyl-CoA levels feedback to inhibit PDH and the ensuing high citrate levels inhibit the glycolytic enzyme, PFK-1. In a range of colon and breast cancer cell lines, the Glucose Fatty Acid or Randle cycle was also detected (Fine et al., 2009). Thus, all the cancer cell lines tested showed parallel reductions in growth and ATP when cultured in media containing the KB, acetoacetate *plus* glucose *versus* glucose media alone.

The extent of the KB effect on growth and ATP levels in cancer cells directly correlates with the levels of expression of the mitochondrial uncoupling protein, UCP-2 and this protein has recently been proposed to function as a metabolic sensor coupling glucose oxidation to mitochondrial metabolism (Pecqueur et al., 2008; 2009). Primary mouse embryonic fibroblasts

(MEFs) with heterozygous deletions of UCP-2 growing in high glucose media without KB grew faster even in low (3%) oxygen and knocking down UCP-2 (UCP^{-/-}) was associated with a metabolic shift to dependency on glucose with increased glycolytic and mitochondrial respiration rates and reduced β -oxidation, ATP/ADP ratios and NADH levels.

Over-expressing UCP-2 in other primary cell lines, including CHO cells, retarded their growth rates. In these normal cell types, UCP-2 expression showed no effect on ROS production after respiratory chain inhibitor (rotenone or antimycin A) treatment, indicating that in the experimental context of normal cell function UCP-2 does not exert its uncoupling (H^+ ionophoretic) activity. The action of UCP-2 in these normal cells seems quite distinct from the well characterised function of the brown adipose tissue protein, UCP-1, which in response to superoxide and fatty acids uncouples the respiratory chain and OxPhos by dissipating the proton gradient across the mitochondrial inner membrane, thereby releasing heat in a futile cycle and inhibiting ATP production (reviewed in Brand et al., 2004; Sluse et al., 2006). By contrast, UCP-2 appears to function as part of a switching mechanism from glycolysis to fatty acid oxidation and the signalling process involved is not entirely clear. Thus, one possibility is that the direct interaction of UCP-2 with particular FA and lipid peroxides produced by ROS action is involved in regulating their function as proton gradient uncouplers (Echtay et al., 2002a,b, 2003; Parker et al., 2008). However, it may also involve AMPK activity (Buzzai et al., 2005) if UCP-2 induction leads to decreased ATP and increased cytosolic AMP which would activate AMPK. Thus, AKT transformed cancer cells where AMPK was activated using the AMP analogue, 5-amino-4-imidazolecarboxamide riboside (AICAR) switched the cells from glucose dependency to fatty acid oxidation for growth (Buzzai et al., 2005).

The results obtained from studies of UCP-2 expression in normal cells contrast markedly with those from studies of UCP-2 in cancer cells (Derdak et al., 2008). In the latter cells, UCP-2 plays a critical role in the adaptive response in cancer cells as a mitochondrial suppressor of ROS, acting as a classical uncoupling protein. UCP-2 expression was increased in a range of drug-

resistant cancer cell lines and human colon cancers. Over-expressing UCP-2 in HCT116 human colon cancer cells inhibited ROS accumulation and apoptosis induced by exposure to cytotoxic chemotherapeutic agents including etoposide, camptothecin and doxorubicin. This protective role was associated with reduced p53 phosphorylation and in this study, it was linked to the induction of a glycolytic phenotype. These results were noted to be consistent with observations in some other reports on the function of UCP-2 in cancer cells (Harper et al., 2002; Horimoto et al., 2004; Collins et al., 2005) but are inconsistent with other studies of UCP-2 function in promoting FA oxidation and they highlight the problems faced in studying mitochondrial metabolic regulation of normal *versus* cancer cells.

Studies of cancer cells must carefully consider the role of glucose/insulin and fatty acid levels supplied as energy substrates and their effects on the levels of UCP-2 and its function. Recently, for example, it was reported that UCP-2 mRNA expression in normal bovine mammary epithelial cells in culture is greatly up-regulated in a dose responsive manner by adding long chain saturated FA, including palmitate and stearate, and particularly the unsaturated FA oleate and linoleate (Yonezawa et al., 2008). Insulin (5-50 ng/ml) significantly suppressed the expression of UCP-2 and given its likely presence in most cell culture media, insulin must also be taken into account when studying UCP function. Many studies have shown that FA's increase cellular triacylglycerol uptake and induce β -oxidative enzymes and UCP gene expression. It is also well established that the induction of UCP-2 gene expression involves PPAR's α or γ (reviewed in Villarroya et al., 2007). In addition, PPAR-independent induction of UCP-2 by the sulphur-substituted fatty acid analogues, tetradecylthioacetic acid (TTA) (Berge et al., 2001) and (4-chloro-6-[(2,3-dimethylphenyl)amino]-2-pyrimidinyl)thioacetic acid (Wy14643) (Zhang et al., 2007) has been reported to directly involve ROS production and oxidative pathways.

The mitochondria are potent producers of superoxide as a by-product of internal oxidative processes such as FA β -oxidation or respiratory chain function. Cancer cells can alter their energy supply by switching from active glycolysis to fatty acid oxidation with an associated increase in

Kreb's cycle activity and OxPhos. An increased rate of electron transport across the respiratory chain leads to a diminished steady-state level of reductive intermediates (FADH₂, ubiquinol, Fe²⁺), the electron donors for superoxide generation. Presumably, the reason for this is to prevent mitochondrial overload, excessive ROS production and apoptosis from occurring. It would appear that mitochondrial overload resulting in excessive ROS production and induction of apoptosis is prevented by high energy substrates (*e.g.* FA's) inducing UCP's as a gearing mechanism that maintains the balance between energy production coupled to respiration *versus* energy dissipation as heat (Echtay et al., 2002a,b), thereby avoiding the critical point of ROS excess triggering cell death. This conclusion is also supported by the observation that methylmalonate (Dutra et al., 1993; Toyoshima et al., 2005) and malonate (Fernandez-Gomez et al., 2005) as key intermediate metabolites of FA metabolism inhibit dicarboxylate transporter and complex II SDH respiratory chain function (Mirandola et al., 2008; in addition, see section 9 below), yet induce ROS leading to apoptosis. Moreover, it has long been known that FA's by themselves can act as mitochondrial uncouplers and inhibitors of pyruvate oxidation (Koga et al., 1984). Hence, these aspects of FA metabolism must be overcome if cancer cells are to survive and this is one function of UCP-2. Samudio et al. (2009) proposed, given that FA oxidation in cancer cells was linked to chemoresistance and mitochondrial uncoupling by UCP that Warburg's observations could result from the preferential oxidation of FA's by cancer cell mitochondria. Hence, targeting FA oxidation or anaplerotic pathways that support FA oxidation could provide a novel cancer therapy (Samudio et al., 2009).

4.4 Targeting fatty acid oxidation

Given the above observations on FA oxidation in cancer cells, is it possible to use this knowledge to develop new targeted anticancer therapies? Carnitine palmitoyltransferase I (CPT I) in mitochondria and carnitine octanoyltransferase (COT) in peroxisomes catalyze the conversion of long- and medium-chain acyl-CoA to acylcarnitines in the presence of carnitine. Both enzymes

are inhibited by the drug, etomoxir which blocks β -oxidation of FA's, induces ROS production and apoptosis of Hep G2 cells, and potentiates the activity of chemotherapeutic anticancer agents such as cisplatin (Hernlund et al., 2008). Other studies on a range of cancer cell types and xenografted tumors including leukemia (Iversen et al., 2006), gliomas (Berge et al., 2003) and colon cancer (Jensen et al., 2007) also support targeting FA oxidation as a cancer therapy. It now remains to be determined whether such inhibitors of mitochondrial β -oxidation will show similar anticancer effects and no severe side-effects in human clinical trials.

5 Glutamine and glutamate oxidation:

Although glutamine is the principal nitrogen donor for nucleotide and amino acid synthesis, its oxidation yields reducing equivalents (NADH, FADH₂), CO₂, H₂O and lactate as end-products in a process commonly named “glutaminolysis”, involving both mitochondrial and cytosolic enzymes (Fig. 1). Glutamine is commonly used in cancer cells as an alternative energy source, particularly when glucose is deficient. Glutaminolysis activation depends on a significant host glutamine supply, which is very actively taken up and transported for consumption by tumor cells. In addition, for full glutamine oxidation, a constant supply of acetyl-CoA derived from other carbon sources (pyruvate, FA, KB) is required to help reduce a rise in the levels of malate by driving conversion of malate into oxaloacetate and then citrate by mitochondrial malate dehydrogenase and citrate synthase, respectively (Fig. 1).

5.1 Plasma membrane glutamine transport

Mice bearing the Lewis lung carcinoma showed a high tumor glutaminase activity and glutamine concentrations in their liver and kidney were 1.7-times lower than that observed in tumor or healthy tissue from normal mice in agreement with an accelerated glutamine consumption by the cells in the tumor (Rivera et al., 1988). However, before the reduced glutamine tissue levels occur, the available glutamine significantly increases as mRNA levels

and activity of kidney and liver glutamine synthetase both increase during cancer progression. The glutamine synthase condenses glutamate and ammonia to increase glutamine levels (Aledo et al., 2000), thereby increasing its availability as an energy source for tumor metabolism, resulting in host cachexia.

Extracellular glutamine is also actively transported into tumor cells by highly efficient plasma membrane glutamine transporters. In mammalian cells, glutamine uptake is mediated by four transport systems, some of them highly sodium-dependent: (a) SNAT1 found in neurons; (b) System N (SNAT3) found in astrocytes, liver, renal proximal tubule and skeletal muscle; (c) System ASC (ASCT2) found in normal proliferating cells and (d) SLC6A19, recently discovered in brush border membranes (McGivan and Bungard, 2007). Hepatoma, glioblastoma, hepatocarcinomas, benign prostatic hyperplasia and prostate adenocarcinoma tumor cells all over-express the ASCT2 isoform (Li et al., 2003; Sidoryk et al., 2004; Fuchs et al., 2004; Fuchs and Bode, 2005), whereas anaplastic astrocytoma and glioblastoma brain tumors also express the SNAT3 isoform (Sidoryk et al., 2004). Furthermore, it has been widely documented that glutamine transport across plasma membranes of fast-growing tumor cells is substantially higher than their normal counterparts (4.5-12 nmol/min/mg protein in lymphocytes and liver *versus* 65 nmol/min/mg protein in HeLa tumor cells) (Jacob et al., 1986; Piva et al., 1992; Piva and McEvoy-Bowe, 1998).

In tumor cells, the cytosolic glutamine has two possible fates, either (i) to be transported into the mitochondria and metabolized (Fig. 1) or (ii) to be utilized for the synthesis of purines, pyrimidines or glutathione (after deamidation to glutamate).

5.2 Mitochondrial glutamine transport

In mammalian cells, the cytosolic glutamine is transported into mitochondria through selective glutamine transporters. In rat liver and kidney mitochondria, glutamine uptake is mediated by a neutral uniport transporter (Schoolwerth and LaNoue, 1985; Soboll et al., 1991),

whereas in rat brain mitochondria, glutamine seems also transported by an electrogenic symporter (with H⁺) and through facilitated diffusion (Kvamme et al., 2000). Using mitochondrial-inner-membrane vesicles from Ehrlich ascites tumor cells it was demonstrated that L-glutamine is transported by a cooperative mitochondrial system with high selectivity for this amino acid and no affinity towards D-glutamine, L-cysteine, L-histidine, L-alanine, L-serine or L-leucine (Molina et al., 1995). Thus, the maximal rate of this transporter was 1.5-3.5-times higher than that of glutamine transport by mitochondria from normal rat liver (Soboll et al., 1991; Molina et al., 1995). This is consistent with an increased transporter expression and with the rapid uptake of glutamine into tumor cell and mitochondria for use as an alternative energy source.

5.3 Glutaminase

Once in the tumor mitochondrial matrix, glutamine is deaminated to form glutamate and ammonia by a very active and over-expressed glutaminase, an enzyme strongly activated by inorganic phosphate (Pi). Two glutaminase isoforms are expressed in normal mammalian cells, which can be identified by their kinetic properties and molecular weights: Kidney- (high affinity for glutamine, low affinity for Pi and strongly inhibited by glutamate) and Liver- (low affinity for glutamine, low activation by Pi, highly activated by ammonium, but not inhibited by glutamate) type glutaminases.

Some malignant tumors (human colorectal, glioma, hepatocarcinoma) over-express the K-type isoform (Linder-Horowitz et al., 1969; Quesada et al., 1988; Turner and McGivan, 2003; Szeliga et al., 2005). However, in other malignant human tumors (leukemia, breast, medullar aplasia) the L-type is the predominant isoform (Pérez-Gómez et al., 2005). Tumor and normal glutaminases (Ehrlich ascites, kidney, liver) are potently activated by Pi. However, Pi stimulation appears to be higher in fast-growing tumor cells (Dunning LC18) than in benign tumors, fetal liver and adult liver but not in adult kidney tissue (Linder-Horowitz et al., 1969).

In particular, the enzyme activity in Ehrlich ascites tumor and AA1 and HT29 colon carcinomas increases by 30-70 times in the presence of Pi concentrations that might be found in their mitochondrial matrix (50-70 mM) (Medina et al., 1988; Quesada et al., 1988; Turner and McGivan, 2003). This last observation may explain the high tumor glutaminase activity found in tumors compared with non-neoplastic tissue (Table 2). For rat kidney and tumor glutaminase, increasing the phosphate concentration from 5 to 100 mM Pi augments the glutamine affinity and the *K_m* for glutamine diminishes from 36 to 4 mM (Shapiro et al., 1982).

Other organic phosphates (glucose-6-phosphate, fructose 6-phosphate, fructose-1,6-bisphosphate, dihydroxyacetone phosphate, or carbamoyl phosphate) and anionic compounds (arsenate, arsenite, borate, succinate, malate, citrate, lactate) do not affect glutaminase enzyme activity (Quesada et al., 1988). Tumor glutaminase is not affected by physiological ammonia concentrations, whereas the liver mitochondrial enzyme is strongly inhibited (Verhoeven et al., 1983).

Glutaminase expression correlates with the development of increased malignancy (Segura et al., 2005). In the highly malignant Duning LC18 tumor the glutaminase expression is substantially increased (8-12 times) above that in benign tumors (Morris 7777, 9633, 9618A) and healthy tissue (fetal liver) (Linder-Horowitz et al., 1969). Thus, glutaminase has been considered as a good cancer prognostic marker. However, further studies are required evaluating the enzyme activity together with tumor malignancy to establish how strict this relationship is.

Relatively little information is available about the regulatory mechanism controlling glutaminase expression. The two glutaminase isoforms are the products of different related genes, the GLS1 (K-type) and GLS2 (L-type) genes. Myc up-regulates GLS1 expression more than 10-fold in the P493-6 human Burkitt's lymphoma (Gao et al., 2009; reviewed in Dang, 2009). Plasma membrane glutamine transporter SLC7 is also up-regulated by Myc whereas glutamate dehydrogenase is not regulated by this oncogene. Glutaminase expression may also be regulated by intermediary metabolites. For example, high butyrate concentrations induced

glutaminase down-regulation (colonic epithelial cells), reduced activity (colonic epithelial cells, SK-Hep tumor cells) (Cherbuy et al., 2004), and also reduced glutamine transport (SK-Hep tumor cells) (Bode and Souba, 1994) (see Butyrate section 9 below for more detail).

When 4-hydroxy-tamoxifen is used to activate estrogen receptor-Myc-immortalized human fibroblasts, glutamine-, but not glucose-, depletion promotes apoptosis. The apoptotic process results from the excessive diminution in the glutamate, 4-aminobutyrate, 5-oxoproline and aspartate pools, the principal products of glutamine metabolism, whereas ATP and glutathione levels remain constant and no DNA damage is detected. These results strongly suggest that glutamine-depletion limits Krebs's cycle flux, which in turn modifies the NADH/NAD⁺ and FADH₂/FADH ratios and hence redox homeostasis. The changes in reducing equivalents and the diminution in other Krebs's cycle intermediates affect gene transcription and induce tumorigenesis (Yuneva et al., 2007).

5.4 Glutamine synthetase

In normal cells, the glutamate/glutamine inter-conversion is catalyzed by glutamine synthetase and glutaminase. However, in fast growth tumor cells, the expression of glutamine synthetase is suppressed or down-regulated (Piva and McEvoy-Bowe, 1998). Thus, in the presence of an over-expressed glutaminase in cancer cells, the prevalent reaction is in the glutamine-to-glutamate direction. However, some tumors such as human breast cancer, may exhibit glutamine synthetase up-regulation as an adaptative response to glutamine depletion (Collins et al., 1997).

5.5 Glutamate dehydrogenase

Glutamate is the substrate of glutamate dehydrogenase, producing 2-oxoglutarate (2OG), a Krebs's cycle intermediate (Fig. 1). In isolated mitochondria from non-tumorigenic tissue and in liver homogenates (Pitot et al., 1960) the activity of glutamate dehydrogenase was higher than in its tumor counterpart (Table 1). However, in biopsies of human hepatocarcinoma, a

substantial increment in glutamate dehydrogenase was observed compared with normal human liver (Glazer et al., 1974). In normal cells, glutamate may also be transaminated to generate 2OG through alanine transaminase. However, low levels of alanine transaminase have been reported in different tumors (Walker carcinoma 256, lymphosarcomas, and sarcoma 180) compared with normal tissues (Harding et al., 1964). Therefore, these observations support the hypothesis that glutamine oxidation through glutaminase and glutamate dehydrogenase is the principal route feeding glutamine into the Krebs's cycle (reviewed in DeBerardinis et al., 2008) and OxPhos to supply ATP (Fig. 1).

The oxidation of glutamine through the Krebs's cycle to oxaloacetate requires acetyl-CoA derived either from glycolysis, β -oxidation of FA or KB oxidation in order to generate the citrate required for the synthesis of essential FA's and cholesterol substrates to form biomembranes, and for full glutamine oxidation via the Krebs's cycle and OxPhos to produce reducing equivalents and ATP (Fig. 1). Therefore, the coexistence of glutaminolysis and glycolysis provides flexibility and vital advantages for tumor proliferation and metastasis. When several carcinomas (HeLa, AS-30D) were cultured in glucose-rich but glutamine-depleted medium, tumor growth became significantly diminished, indicating that glutamine oxidation supplies ATP, and intermediates for anabolic pathways (Rodríguez-Enríquez et al., 2006; Drogat et al., 2007; Donadio et al., 2008). Several other studies have also shown the adaptability of cancer cells to switch to using either glucose or glutamine interchangeably as alternative substrates for oxidative growth (Reitzer et al., 1979; Baggetto, 1992; Wehrle et al., 2000; Rossignol et al., 2004).

5.6 NADP⁺-dependent Malic Enzyme and Lactate dehydrogenase

In parallel with citrate formation, tumor glutaminolysis increases the cellular reducing-equivalents (NADH, NADPH) required for anabolic processes and for electron transport through the respiratory chain (Fig. 1). The mitochondrial malate derived from glutamine oxidation in the

Kreb's cycle can be actively expelled to the cytosol and rapidly oxidized to pyruvate by a very active and over-expressed cytosolic NADP⁺-dependent malic enzyme with concomitant NADPH formation (Fig. 1, Table 2). In some tumor cells (AS-30D, Ehrlich, P1798 thymoma and L1210 leukemia), malate can also be actively oxidized by a mitochondrial NADP⁺-dependent malic enzyme also generating NADPH and mitochondrial pyruvate to directly feed the PDH complex and enter the Kreb's cycle (Moreadith and Lehninger, 1984). The activity of the mitochondrial malic enzyme is regulated by ATP as an active site inhibitor, competing for NAD⁺, and by fumarate as an allosteric activator (Yang et al., 2002). This regulation may be a crucial aspect of the control of tumor glutaminolysis, as ATP is the ultimate product, and fumarate is the product from the previous step of the glutamine metabolic pathway in the Kreb's cycle (Fig.1).

In fact, it has been demonstrated in some fast-growing glioblastomas that the glutaminolysis-derived NADPH may bypass the FA biosynthesis requirements, suggesting that other synthetic pathways such as nucleotide synthesis and glutathione maintenance are also favoured (reviewed in DeBerardinis et al., 2008). Cytosolic pyruvate formed by malic enzyme (Fig. 1) is a substrate for HIF-1 α induced LDH, particularly subtype A (reviewed in Marin-Hernandez et al., 2009).

5.7 Importance of glutamine in tumor metabolism

Glutamine is an anapleurotic precursor and source of NADPH, carbon skeletons and nitrogen. Glutaminolysis provides reducing equivalents in the form of NADPH *via* the reactions catalyzed by glutamate dehydrogenase, isocitrate dehydrogenase and malic enzyme (NADP⁺ malate dehydrogenase) as long as glutamine is available. However, glutaminolysis also provides (a) lactate and alanine (Fig. 1) which may be used as precursors for hepatic gluconeogenesis, providing more fuel for tumor metabolism (reviewed in DeBerardinis et al., 2009); (b) citrate for lipid biosynthesis; (c) ATP by OxPhos (driven by the reducing equivalents formed by 2OGDH and SDH); (d) succinate, 2OG and fumarate which contribute to regulating gene transcription

and/or tumorigenesis; and (e) pyruvate and oxaloacetate acting as antioxidants (reviewed in Yuneva et al., 2007). This reflects a specialized form of metabolism that will self-sufficiently enable tumor growth and proliferation. Therefore, in cancer, glutamine metabolism (including glutaminase and malic enzyme as the two enzymes with higher activity compared with normal cells (see Table 2), and the plasma membrane glutamine transporter ASCT2, which is presumably only expressed in tumor cells could provide alternative and specific targets for cancer therapy.

A range of clinical trials were carried out from 1980-1990 using the drug, acivicin, which is an antibiotic antagonistic to glutamine with potent oncolytic activity (Poster et al., 1981; Bonomi et al., 1994; Hidalgo et al., 1998). However, the drug induced CNS toxicity, was not considered very effective, and resistance to the drug frequently occurred by restricted uptake (Jayaram et al., 1985), presumably as a result of multidrug resistance (MDR) gene expression by the cancer cells. Another promising glutamine-analogue drug was DON (6-diazo-5-oxo-l-norleucine) but, similar to acivicin, although found to be effective in animal models, it proved unacceptably toxic in clinical trials (reviewed in Ahluwalia et al., 1990). More recent studies using antisense-suppression of glutaminase in Ehrlich ascites or SCT2 in human hepatoma confirmed the decreased proliferation and tumorigenicity of both cancer cells and activation of caspase-induced apoptosis in the hepatoma, once again revealing an important role for this enzyme and the oxidation pathway in tumor development (Lobo et al., 2000; Fuchs et al., 2004). Nevertheless, further pursuit of stand alone strategies targeting glutamine pathways as alternative energy sources for cancer therapy does not appear to be favoured as there has been little recent output in this area, possibly because of problems related to neurotoxicity.

5.8 Proline oxidation as an alternative supply of glutamate

Proline provides an alternative source of glutamate for use by tumor cells in the absence of other energy substrates for growth when conditions of nutrient or energy stress require

catabolic energy generating processes. This situation most likely arises in tumor microenvironments (reviewed in Phang et al., 2008). Catabolising proline provides one survival mechanism as proline can feed into cellular bioenergetic pathways. Proline unlike other amino acids is unique, lacking the primary α -amino group and therefore is not metabolized by transamination or decarboxylation. In addition, proline is one of the most abundant amino acids and more importantly it is a large constituent of proteins in the cellular microenvironment. The extracellular matrix (ECM) contains an abundance of collagen with its high content of proline and hydroxyproline. As the surrounding ECM is degraded by matrix metalloproteinases during tumor progression (for example, see Li et al., 2006), proline is released and used for protein synthesis or oxidized in the mitochondria for energy production. Proline oxidase (POX), a flavoenzyme localized at the inner mitochondrial membrane, catalyzes the first step of proline degradation by converting proline to Δ -pyrroline-5-carboxylate (P5C). P5C is then non-enzymatically transformed into glutamate semialdehyde before dehydrogenation by P5C dehydrogenase to form glutamate (Valle et al., 1979; McKnight and Hird, 1986; Pandhare et al., 2009) and hence to produce 2-OG, thereby feeding into the Krebs's cycle (Fig. 1).

Proline oxidation likely contributes to ATP production, important for survival under conditions where other nutrients are lacking (Phang, 1985) and increased activity of proline oxidase has been detected in malignantly transformed fibroblasts (Downing et al., 1977). However, proline oxidase is a p53-induced redox gene that produces ROS and will induce apoptosis in tumor cells if sufficiently active (Rivera and Maxwell, 2005). During p53-induced apoptosis POX levels are markedly increased, resulting in the proline-dependent production of ROS, specifically superoxide radicals leading to apoptosis (Liu et al., 2006; 2008; 2009). Fast-growing malignant renal carcinomas with mutant p53 have severely reduced levels of POX compared to normal tissue (Maxwell and Rivera, 2003).

In mitochondria, proline and hydroxyproline are oxidized to yield P5C and OH-P5C, by POX and hydroxyproline oxidase (OH-POX), respectively, and these two intermediates can be

converted to glutamate and γ -hydroxy glutamate by the same enzyme, P5C dehydrogenase. OH-POX is not normally present in the human Lovo and RKO colon carcinoma cell lines but adriamycin (doxorubicin) treatment increased the levels of OH-POX, promoting Krebs' cycle entry and production of pyruvate. Similar to POX, hydroxyproline oxidase levels are regulated by p53 and OH-POX function like POX was also associated with mitochondrial ROS generation and apoptosis (Cooper et al., 2008). Thus, the two enzymes, POX and OH-POX are analogous in that both are capable of providing alternative energy substrates for cancer cells under metabolic stress. Another important energy generating mechanism when glucose is limited involves proline cycling between mitochondria and the cytosol and P5C reductase which participates in a metabolic interlock with glucose-6-phosphate dehydrogenase of the pentose phosphate pathway to transfer reducing equivalents as NADPH/NADH into mitochondria to produce ATP (reviewed in Phang et al., 2008). Therefore, proline and hydroxyproline may fuel bioenergetics not only by supplying carbons to the Krebs' cycle but also by the recruitment of the pentose phosphate pathway linked by the proline cycle.

Although proline degradation may be an inefficient source for ATP production, its contribution could be significant in the metabolic adaptation of cancer cells to nutrient depletion (Pandhare et al., 2009). Furthermore, under nutrient stress, the oxidation of proline is channelled toward ATP production. However, if survival becomes impossible, a switch is activated to couple the oxidation of proline to the generation of ROS for apoptosis. No studies have been undertaken as to whether or not POX, or OH-POX, provides useful targets for cancer therapy.

6. Peroxisome Proliferator-Activator Receptors (PPARs)

Three different isoforms of PPARs have been described, which are encoded by three different genes, PPAR α , PPAR β/δ , and PPAR γ (reviewed in Li and Palinski, 2006). There are two PPAR γ isoforms, PPAR γ 1 and PPAR γ 2 (with 28 residues more in the N-terminal region), which result from alternative splicing of the same gene. PPAR isoforms show tissue-specific expression

and regulate the expression of genes encoding enzymes involved in glucose and lipid metabolism. PPAR's regulate mobilization, storage, capture, intracellular transport and oxidation of lipids. Several genes encoding transporters and enzymes of the three FA oxidation systems in liver are up-regulated by PPAR's. For mitochondrial β -oxidation, the PPAR regulated genes include the mitochondrial acyl-CoA dehydrogenases VLCAD, LCAD, SCAD (an exception being the medium-chain specific enzyme), short-chain 3-ketoacyl-CoA thiolase (which is also required for degradation of KB) (Aoyama et al., 1998), and malonyl-CoA decarboxylase (Campbell et al., 2002).

Selective pharmacological activation of the PPAR isoforms has provided insight into their cellular roles. Thus, the fibrate drugs used currently in the treatment of dyslipidemia, are synthetic ligands for PPAR α , whereas thiazolidinediones or glitazones preferentially bind PPAR γ and are used as hypoglycemic agents. PPAR γ is over-expressed in prostate, colon, breast, non-small cell lung, and brain cancers (Kubota et al., 1998; Sarraf et al., 1998; Elstner et al., 1998; Chang and Szabo, 2000) and appears to be involved in growth control, induction of apoptosis and differentiation of many types of cancer cells. The PPAR γ synthetic agonists troglitazone, ciglitazone, rosiglitazone and GW7845 (Fig. 3) induced growth arrest and apoptosis of several cancer cell types (Kobota et al., 1998; Sarraf et al., 1998; Elstner et al., 1998; Chang and Szabo, 2000; Morosetti et al., 2004; reviewed in Koeffler, 2003). Natural and synthetic PPAR γ ligands also induced suppression of growth and cause apoptosis in glioblastoma cell lines that express higher PPAR γ levels than normal brain (Morosetti et al., 2004). Troglitazone induced over-expression of PPAR γ , 3-hydroxy-3-methylglutaryl-CoA synthetase (HMGCS), HMGC reductase, FAS, and stimulation of mitochondrial β -oxidation in lung alveolar carcinoma (Andela et al., 2005).

These observations suggest that by inducing increased FA oxidation and decreased glucose oxidation, PPAR γ ligands arrest cell growth and promote apoptosis of cancer cells, although the precise mechanisms are unclear. As described in section 4.2, the omega-3 (or n-3) (PUFA's and

their metabolites are natural ligands for PPAR γ and act as anticancer agents (reviewed in Edwards and O'Flaherty, 2008). Dietary intake of omega-3 PUFA's is associated with reduced risk of certain cancers in humans and animals. *In vitro* studies have shown that omega-3 PUFA's inhibit cell proliferation and induce apoptosis in cancer cells through various pathways, one of which involves PPAR γ activation. The differential activation of PPAR γ and PPAR γ -regulated genes by specific dietary fatty acids may be central to their distinct roles in cancer and perhaps its treatment as well.

The specific mechanism of PPAR induced cancer cell death remains unclear and building theories based only on transcriptional profiling, without assessing biochemical function, whilst informative, does not provide proper understanding of the energy metabolism of cancer cells and the best way to control it to kill cancer cells, particularly by affecting mitochondrial function and ROS production. A connection between PPAR γ and proline oxidase (POX – see section 5.8 above) has been made in that the apoptotic action of PPAR γ activation in human non-small cell lung cancer was shown to be mediated *via* POX induced ROS production (Kim et al, 2007; reviewed in Phang et al, 2008). Unfortunately, the field abounds with studies based solely on molecular biological approaches. For example, a PPAR γ -independent mechanism was proposed to explain the effects of glitazones on tumor growth by using PPAR $\gamma^{-/-}$ cell lines (Palakurthi et al., 2001; see also Koeffler, 2003). Clearly, further experimentation is needed to clarify this area.

Elucidating the role of PPAR's in cancer is made additionally complex by observations that most of the PPAR binding drugs, including fibrates and glitazones, at pharmacological relevant levels also directly affect mitochondrial respiratory chain function, acting in a PPAR independent manner (reviewed in Scatena et al., 2008). For example, in HepG2 hepatocarcinoma cells, the PPAR- γ ligand ciglitazone (Fig. 3) caused a dose-dependent inhibition of complex I by about 60% at a drug concentration of 50 μ M, associated with similar reductions in growth and increased cytotoxic effects (Scatena et al., 2007a; 2007b). The fibrates were much weaker in their effect requiring near millimolar levels.

A series of compensatory metabolic adaptations occurs in cancer cells treated with PPAR agonists as demonstrated by significant increases in lactate, acetate, pyruvate and alanine concentrations measured in cell supernatants. These results suggest that a significant shift of cell metabolism occurs in order to compensate for a decreased OxPhos and Krebs' cycle. In other words, the partial failure of the Krebs' cycle is partially counterbalanced by the stimulation of other energy producing pathways, including anaerobic glycolysis. Thus, Scatena et al. (2008) proposed that the impairment of NADH oxidation by inhibiting respiratory complex I can drive cells to switch their metabolism to both anaerobic glycolysis and β -oxidation. This metabolic switch occurred by inhibiting NADH synthesis while stimulating FADH₂ production, in order to engage the remaining active energy producing components of the mitochondria, including the respiratory chain, glycerol catabolism and mitochondrial FAD-dependent glycerol-3-phosphate dehydrogenase and/or fatty acid β -oxidation via electron-transferring flavoprotein (Scatena et al., 2008). These mechanisms could contribute to the strong hypotrygliceridemic activity and light hypoglycemic effect of PPAR agonists.

7. AKT/AMPK and the switch from glucose to alternative energy substrates by cancer cells.

The dogmatic view of a prevalent glycolytic phenotype in cancer cells is especially dominant in the field of brain tumors. However, studies on the dependence on glycolysis of several glioma cell lines have revealed that some of them are highly tolerant to glucose deprivation whereas other gliomas are extremely sensitive, although the latter can be rescued from glucose deprivation by adding alternative oxidizable (mitochondrial) substrates (Griguer et al., 2005). These observations suggest the presence of very active mitochondrial function in brain tumors reflecting a high requirement for ATP (see Table 1).

Glioblastoma is the most common malignant tumor of the central nervous system and patients with this cancer have a very poor prognosis which appears to be related to the expression of the *Akt* gene. AKT is an oncoprotein involved in the PI3K/AKT/mTOR cellular signaling

pathways that promote cell survival by inhibiting apoptotic processes, helping prevent cell death during growth factor deprivation (reviewed in Miyamoto et al., 2009). AKT, one of the most frequently activated Ser/Thr protein kinases in human cancers induces the glycolytic phenotype in cancer cells by phosphorylating hexokinase II to increase its association with VDAC on the mitochondrial outer membrane, providing direct access to mitochondrially produced ATP (reviewed in Ralph et al., 2006; Robey and Hay, 2009). The actions of AKT render cells dependent on the availability of glucose for survival. In a study of glioblastoma cells Buzzai et al. (2005) found, paradoxically, that cells expressing constitutively active forms of AKT became sensitized to undergo apoptosis when deprived of glucose.

By contrast, activating AMPK in the glioblastoma cells with constitutively active AKT using AICAR allowed the cells to survive glucose deprivation because they were able to up-regulate FA oxidation and suppress lipid synthesis. The authors concluded that cancer cells with active AKT were impaired in their ability to induce FA oxidation in response to glucose deprivation unless AMPK was stimulated, enabling survival (Buzzai et al., 2005). In a recent study of EGFR-activated glioblastoma cell lines, AICAR was a more effective inhibitor of cell growth than the mTOR pathway inhibitor, rapamycin (Guo et al., 2009). AICAR induced growth inhibition even though AMPK activation was not as efficient as rapamycin at inhibiting the mTOR signalling pathway. It was concluded that AMPK acted not only by affecting the mTOR pathway but more importantly by modifying the cellular FA catabolism. This was further supported by the observation that adding metabolic products of cholesterol and FA synthesis rescued the growth inhibitory effect of AICAR, whereas inhibiting lipogenic enzymes mimicked AMPK activation. These results demonstrated that AMPK blocked tumor cell proliferation primarily through inhibition of cholesterol and FA synthesis. Most importantly, AICAR treatment in mice significantly inhibited the growth and glycolysis of glioblastoma xenografts engineered to express EGFR ν III, but not their parental counterparts (Guo et al., 2009). Hence, from the data above, it is

suggested that AMPK acts as a switch, changing cellular energy metabolism away from glycolysis to favour FA oxidation as a means for providing sufficient ATP by OxPhos for growth.

AMPK inhibits FA synthesis in cancer cells by phosphorylating and inhibiting HMG-CoA reductase and ACAC (of which there are two isoforms, ACAC1 and ACAC2) (reviewed in Hardie and Pan, 2002). ACAC2 is anchored to the mitochondrial membrane, co-localized with CPT-I (Abu-Elheiga et al., 2000). ACAC2 inactivation by AMPK leads to diminished levels of malonyl-CoA, an inhibitor of CPT-I. As a result, increased palmitoyl transfer into the mitochondrial matrix occurs, promoting FA β -oxidation and utilization of FA as energy sources (also see section 4.2 and 4.3 for more detail).

Several studies have shown that AKT activity in cancer cells opposes the effects of AMPK activity and hence these two opposing forces act as a regulatory switch altering the balance of energy flows in cancer cells between glycolysis and alternatives like FA β -oxidation. AKT phosphorylates and activates ATP-citrate lyase, increasing acetyl-CoA and malonyl-CoA levels which in turn, inhibits FA oxidation and increases FA synthesis. Hahn-Windgassen et al. (2005) established that AKT was a key regulator of energy metabolism that inhibited AMPK. AKT-deficient cells showed reduced ATP levels and elevated AMPK activity, whereas cells expressing activated AKT had markedly elevated ATP levels and reduced AMPK activity. AKT elevates ATP production via increased glycolysis and OxPhos supported by pyruvate oxidation (Gottlob et al., 2001). The lower ATP level attained in AKT-deficient cells may result from OxPhos supported by FA oxidation that, at the same time, is partially inhibited by the uncoupling effect exerted by the increased mobilization of free FA. AKT activates glycolysis by increasing glucose transporter expression and translocation to the cell membrane, and increased activity and expression of glycolytic enzymes (reviewed in Robey and Hay, 2009). The AKT induced increase in ATP levels causes a concomitant reduction in AMP and thereby reduces AMPK activity. Unfortunately, rates of peroxisomal β -oxidation, glutamine degradation, ATP synthesis or respiration have not been extensively examined and compared in AKT-dependent *versus* -

independent tumor cells. It should be noted that the changes in energy metabolism regulated by AKT and AMPK involve mitochondrial oxidative pathways and OxPhos. Evaluation of these other mitochondrial oxidative pathways should help to establish exactly how and where cancer cells are able to compensate for the lack of glycolytic intermediates and associated reduced levels of cytosolic ATP.

8. Ketone Body (KB) Metabolism

KB metabolism refers to use of compounds including acetoacetate, acetone and β -hydroxybutyrate, mainly produced and secreted by the liver into the bloodstream to other tissues as alternative fuels. They are made from acetyl-CoA to form acetoacetate which can then be converted into β -hydroxybutyrate by β -hydroxybutyrate dehydrogenase (BHBDH). Their metabolism is best characterised in brain cells and it is discussed first before assessing the role of KB utilization in cancer cells. The predominant blood KB is β -hydroxybutyrate which is rapidly oxidized by the reverse reaction of BHBDH to acetoacetate and converted to acetyl-CoA in the mitochondria (Fig. 2). Normally, brain cells only metabolize glucose for energy and pyruvate is predominantly converted to acetyl-CoA in mitochondria of neurons and glia to feed the Krebs cycle with only 13% of pyruvate transformed into lactate in the brain. FA's bound to lipoproteins do not freely pass the blood-brain barrier, although octanoate and other short-chain FA's may be an exception (reviewed in Seyfried and Mukherjee, 2005). Thus, short-chain FA's and KB's might be able to freely diffuse across biological membranes when protonated. In addition, KB's are able to cross the blood-brain barrier and are internalized into neurons and astrocytes via the monocarboxylate transporters, which catalyze the co-transport of H^+ and lactate, pyruvate, KB's or other monocarboxylate molecules. Monocarboxylate transporters catalyzing the co-transport of KB's, lactate or other monocarboxylates, including butyrate, and Na^+ have been identified (Martin et al., 2006; reviewed in Ganapathy et al., 2008). As the pK values of the carboxylic group ionization are around 4, only about 1 FA or KB molecule is protonated per 1000 negatively

charged molecules at pH 7. As a result, transport and diffusion will not be rate-limiting given the rate of ionization is extremely fast and that the presence of transporters ensures rapid flow of short-chain FA's or KB's into the brain. During states of fasting, starvation or caloric restriction when glucose is reduced, brain cells readily metabolize KB's (reviewed in Greene et al., 2003).

In contrast to normal brain cells, gliomas and many other tumor cells lack the metabolic versatility to utilize KB's, showing greater dependency on glycolysis. Moreover, dietary caloric restriction, but not a ketogenic diet (with high fat/low carbohydrate intake) was shown to drastically reduce the growth of astrocytomas (Mukherjee et al., 2002; Seyfried and Mukherjee, 2005) presumably because of diminished glycolysis in tumor cells and increased oxidation of KB's restoring mitochondrial respiratory function. Obviously, further analysis of the relative content and activity of enzymes involved in KB degradation in brain cancers is required as it has not been rigorously evaluated.

Some neuroblastomas, astrocytomas and gliomas metabolize KB's for energy and lipid synthesis (Patel et al., 1981; Roeder et al., 1982) as these tumors show high levels of activity of the three enzymes involved in KB oxidation (Fig. 2). Thus, succinyl-CoA acetoacetyl transferase (SCAAT), the enzyme that initiates KB oxidation and is the presumed rate-limiting step, is 40-fold more active in AS-30D hepatocarcinoma cells than in hepatocytes (Briscoe et al., 1994), whereas in brain tumors a lower, but still significant SCAAT activity is detected in comparison to normal brain tissues (Fredericks and Ramsey, 1978).

Mitochondrial hydroxymethylglutaryl-CoA synthase 2 (HMGCS2) which uses acetoacetyl-CoA to produce HMG-CoA is a possible rate controlling step for KB synthesis (Fig. 2). HMGCS2 is expressed in liver, colon, testis, ovary, pancreas, heart, intestine, kidney and brain. Myc represses HMGCS2 transcription and human HMGCS2 expression is down-regulated in 90% of Myc-dependent colon and rectal tumors, and in 80% of small intestine Myc-independent tumors. Apparently, ketogenesis is an undesirable metabolic characteristic of proliferating cells. Increased

expression and activity of HMGCS2 is induced by high fat diets, PPAR agonists and butyrate (Cherbuy et al., 2004; Camarero et al., 2006) whereas insulin causes the opposite effect.

9. Short Chain Carboxylic acids, Propionate, Acetate and Butyrate

Acetate, propionate and butyrate are generated at high levels by colonic bacterial degradation of unabsorbed starch and non-starch polysaccharides and account for a major fraction of energy consumption by epithelial cells (reviewed in Wong et al., 2006; Hijova and Chmelarova, 2007). Several studies have reported utilization of propionate as a favoured energy substrate by heart cells with anaplerotic rates from 0.25 mM propionate estimated to account for 6-8% of the Krebs's cycle turnover (Kasumov et al., 2007) and other estimates as high as 29% (Sherry et al., 1988). Propionate was shown to be readily utilised as a substrate by Caco-2 colon cancer cells (Malaisse et al., 1996) and was also readily used by the murine mammary carcinoma/sarcoma EMT6 spheroids as an anapleurotic substrate. In the tumor spheroids, propionate was converted to methylmalonate and succinate even in the presence of normal levels of glucose and glutamine in the media (Wehrle et al., 2000). Hence, although use of propionate by tumors *in vivo* has not been extensively studied, the above data indicates that tumor cells are highly likely to metabolise propionate.

The metabolism of propionic acid begins with its conversion to propionyl-CoA, as the common first step in the metabolism of carboxylic acids. Several studies of different mammalian tissues have reported the biochemical characterization of mitochondrial propionyl-CoA synthetases (PCAS) that convert propionate into propionyl-CoA identifying one with a MW of 72kD (Groot, 1976; Ricks and Cook, 1981). The more characterized bacterial gene encoding PCAS, PrpE, is highly homologous to acetyl-CoA synthetase of which there are several isoforms in humans (ACSS1-3). However, the relationship of mammalian mitochondrial PCAS(s) to the family of acetyl-CoA synthetase genes (ACSS 1-3) so far identified is not clear. The ACSS2 enzyme with the closest MW of 68 kD to the characterised PCAS (72kD) is a reversible cytosolic enzyme in

tumor cells, forming acetate from acetyl-CoA and ACSS2 expression is increased in tumor cells under hypoxia (Yoshii et al., 2009b).

Propionyl-CoA is also formed through the catabolism of the branched chain amino acids (BCAAs) isoleucine, valine, methionine and threonine or odd long chain FA and the side chain of cholesterol (Fig. 4). During the catabolism of valine, thymine and compounds (including pyrimidines) catabolized by way of alanine, malonate semialdehyde and methylmalonate semialdehyde are formed. Both of these are used by the same intramitochondrial enzyme, methylmalonate semialdehyde dehydrogenase (MMSDH), the only aldehyde dehydrogenase known to require CoA. MMSDH catalyzes the irreversible oxidative decarboxylation of malonate and methylmalonate semialdehydes to acetyl- and propionyl-CoA, respectively.

Propionyl-CoA cannot be used directly for β -oxidation or by the Krebs's cycle. Propionyl-CoA must first be carboxylated by propionyl-CoA carboxylase (PCAC) to D-methylmalonyl-CoA. PCAC is a mitochondrial biotin-dependent enzyme. Mutations in the genes encoding the PCAC subunits causes an inherited metabolic disease, propionic acidemia, which can be life threatening in the neonatal period. This inborn error of metabolism causes accumulation of propionic acid in the circulation which results in neurodegeneration, mental retardation and motor impairment due to mitochondrial accumulation of propionyl-CoA and its derivative, methylcitrate as two Krebs's cycle inhibitors (reviewed in Ballhausen et al., 2009).

D-methylmalonyl-CoA is isomerized to L-methylmalonyl-CoA by methylmalonyl CoA racemase before methylmalonyl-CoA mutase (MCAM) converts L-methylmalonyl-CoA to succinyl-CoA, requiring 5'-deoxyadenosylcobalamin (AdoCbl) as a cofactor (Fig. 4). MCAM is encoded by the MUT gene whose inborn errors can cause methylmalonic aciduria which similar to propionic aciduria is potentially lethal. Neurological symptoms are frequently found in patients with methylmalonic and propionic acidurias. MCAM, as a vitamin B12-dependent enzyme, is also defective when dietary intake of vitamin B12 is lacking (reviewed in Fowler et al., 2008). The accumulation of propionyl-CoA, methylmalonyl-CoA, malonate or methylmalonate as toxic

metabolites impairs OxPhos resulting in a metabolic stroke (Narasimhan et al., 1996; Trinh et al., 2001). The inhibitory effect of these toxic metabolites on mitochondrial function helps explain the neurotoxicity and vulnerability of brain regions which have high rates of aerobic (mitochondrial) metabolism such as in the basal ganglia [Ballhausen et al., 2009]. It is worth noting that these effects on mitochondria account for the tumor selective action of targeted inhibitors of FA metabolism (see sections 4.1-4.2 above).

Alternatively, methylmalonate and malonate as tumor mitochondrial toxins can be produced from methylmalonyl-CoA and malonyl-CoA respectively (Fig. 4), through deacetylation and acetylation of free (methyl) malonic acid as intermediate by-products of the mitochondrial MM-CoA epimerase reaction (Montgomery et al., 1983; Bikker et al., 2006). Three related mechanisms were proposed to explain how methylmalonate and propionic aciduria caused neurological damage (Ballhausen et al., 2009) and these all resemble closely the same situation when FA synthesis is inhibited in cancer cells resulting in their death (see section 4.1 above). The three mechanisms are reiterated here because of their likely importance as mechanism for killing cancer cells:

The “toxic metabolite hypothesis”: Methylmalonate (MMA, the key metabolite of methylmalonic aciduria), was initially considered as a toxic metabolite before other studies reported toxic effects of propionyl-CoA and 2-methylcitrate as well (Okun et al., 2002; Kölker et al., 2003). As discussed in section 4.1, MMA and malonate are known inhibitors of respiratory chain complex II. MMA competitively inhibited the SDH enzyme activity with K_i values of 4.5 and 2.3 μM in brain and liver, respectively (Dutra et al., 1993), causing mitochondrial damage (Narasimham et al., 1996; Wajner and Coelho, 1997). Toxic effects of MMA on primary neuronal cultures and in rats after intra-striatal administration have been shown and could be prevented by succinate, N-methyl-D-aspartate receptor antagonists and antioxidants (Okun et al., 2002).

Synergistic inhibition of mitochondrial energy metabolism: MMA loading in cultured rat striatal neurons results in intracellular accumulation not only of MMA, but also of 2-methylcitrate

and malonate (Okun et al., 2002). Thus, impairment of energy metabolism might be mediated by a synergistic inhibition of the Krebs's cycle and the mitochondrial respiratory chain by 2-methylcitrate, malonate and/or propionyl-CoA (Kölker et al., 2003). OxPhos studies in muscle tissue of propionic aciduria patients showed severely decreased activity of complexes I–IV and mtDNA depletion (Schwab et al., 2006).

Dicarboxylic acids and the “trapping hypothesis”: The blood–brain barrier has a limited transport capacity for dicarboxylates (Sauer et al., 2006). It has been hypothesized that brain-generated dicarboxylates might be trapped in CNS and cause neurodegeneration in organic acidurias. It has also been assumed that MMA might interfere with the transport of dicarboxylates between neurons and astrocytes (Kölker et al., 2003; Kölker and Okun, 2005) and MMA levels are increased in the brain because dicarboxylic acids tend to be trapped inside neural cells (Hoffmann et al., 1993; Sauer et al., 2006). This is further supported by the observation that MMA in the millimolar range inhibited the mitochondrial dicarboxylate transporter, preventing succinate uptake with a significant and dose-dependent inhibition of mitochondrial respiration driven by succinate (Fig. 4). In addition, MMA was shown not to inhibit complex II SDH activity whereas micromolar levels of malonate were inhibitory (Kölker et al., 2003; Mirandola et al., 2008).

Butyrate, but not acetate or other short-chain carboxylic acids, stimulates proliferation of normal colonic epithelial cells and protects them from Bax-mediated apoptosis. In contrast, butyrate reduces proliferation and induces apoptosis in colon cancer cells (Heerdt et al., 1994; Hass et al., 1997) because HMGCS2 is down-regulated in cancer cells and they apparently lose the ability to metabolize butyrate (Camarero et al., 2006). Also, the Na⁺-dependent monocarboxylate transporter required to internalize butyrate is not expressed in cancer cells (Ganapathy et al., 2008). However, in the human colon adenocarcinoma cell line HT-29, butyrate is accumulated and oxidized, albeit at a rate 70% lower than by normal colonocytes and without producing KB or affecting OxPhos (*i.e.*, oligomycin-sensitive respiration) (Leschelle et al., 2000).

A small, but significant amount of administered butyrate (5%) is detected in histones because butyrate may serve as a precursor for histone acetylation while inhibition of nuclear deacetylase by butyrate is also involved in its anti-proliferative effect on human cancer cells (Andriamihaja et al., 2009). Both mechanisms appear to be involved in the butyrate modulation of gene expression. Recently, it was also shown that regulation of cellular propionyl-CoA occurs by propionylation of PrpE and parallels regulation of acetyl-CoA by acetylation of acetyl-CoA synthetase, raising the possibility that propionylation may serve as a regulatory modification in higher organisms (Garrity et al., 2007). Histone propionylation might be generated by the same set of enzymes as for histone acetylation and the selection of donor molecules (propionyl-CoA *versus* acetyl-CoA) may determine the difference in modification (Liu et al., 2009). Because like acetyl-CoA, propionyl-CoA is an important intermediate in biosynthesis and energy production, histone H3 Lys(23) propionylation may provide a novel epigenetic regulatory marker for changes in gene expression affecting cell metabolism in tumors (Liu et al., 2009).

Malate induces increased butyrate oxidation (Fig. 2) and a decrease in its growth-inhibitory effect. Addition of malate drives the formation of oxaloacetate which condenses with acetyl-CoA, derived from butyrate oxidation, to form citrate (Fig. 1). The increased butyrate oxidation promoted by malate lowers butyrate availability for the nucleus. These observations imply that interference with butyrate oxidation modifies butyrate effects on cell growth (Andriamihaja et al., 2009) and that butyrate effects on cancer cells are only indirectly mediated by modulating mitochondrial oxidative metabolism.

Tumor cells expressed higher levels of cytosolic acetyl-CoA synthetase (ACSS2) under hypoxia than normoxia and knockdown of ACSS2 by RNA interference in tumor cells enhanced tumor cell death under long-term hypoxia *in vitro* (Yoshii et al., 2009a). It was also demonstrated that the ACSS2 suppression slowed tumor growth *in vivo*. ACSS2 levels increase in hypoxia and are associated with increased ¹⁴C-acetate uptake whereas knockdown led to a corresponding reduction in acetate uptake with incorporated carbon-14 mostly distributed in the lipid-soluble

fractions in all tumor cell lines examined under normoxia and hypoxia (Yoshii et al., 2009b). Analysis of human hepatocarcinoma cells (HCC) for the patterns of ^{18}F FDG (radiolabelled glucose) and ^{11}C -acetate uptake showed that they complemented each other in both human HCC in patients by PET/CT scanning and in HCC cell lines (Yun et al., 2009). FAS expression was seen in cells with either high ^{18}F FDG or ^{11}C -acetate uptake, suggesting glucose- or acetate-dependent lipid synthesis. Thus, it was concluded that both the cytosolic ACCS2 and mitochondrial ACCS1 expression was important in acetate uptake and acetate-dependent lipid synthesis for the growth of cancer cells with a low-glycolysis phenotype, with glucose or acetate providing compensatory/alternative carbon sources for lipid synthesis in cancer (Yoshii et al., 2009a; 2009b).

To summarise at this point, the metabolism of short chain carboxylic acids has been proposed as yet another putative target for cancer therapy (Yoshii et al., 2009a; 2009b) that should destabilize mitochondrial function. However, very limited information exists where this approach has been tested for treating cancers in either animal models or humans.

10. Dihydroorotate Dehydrogenase (DHODH) and Pyrimidine Biosynthesis.

DHODH is a ubiquitous FMN flavoenzyme thought to be the rate limiting step in *de novo* pyrimidine synthesis and it is the only enzyme in this pathway containing an N-terminal localisation signal that restricts it to the inner membrane/matrix side of mitochondria (Nagy et al., 1992; Rawls et al., 2000). It also contains a hydrophobic channel capable of binding ubiquinone (Ullrich et al., 2001; Heikkila et al., 2007). DHODH is coupled to the respiratory chain in association with complex II SDH at a ratio of 1:4 (Loffler et al., 1996). The enzyme oxidises dihydroorotate to orotate in a reaction that is linked to the respiratory chain via the ubiquinone pool (Fig. 2). Two specific inhibitors used for rheumatoid arthritis treatment, leflunomide ($K_i = 1 \mu\text{M}$; Knecht et al., 1996) and brequinar ($\text{IC}_{50} \sim 0.5 \mu\text{M}$; Cleaveland et al., 1996) interfere with the transfer of electrons from the flavin in DHODH to ubiquinone (Knecht and Loffler, 1998; Bader et al., 1998). However, extensive trials using brequinar for cancer therapy have revealed very little

benefit (for example, see Natale et al., 1992; Cody et al., 1993). Leflunomide (A771726) has recently shown efficacy in killing multiple myeloma cells (Baumann et al., 2009). This probably reflects their B cell origin and dependency on pyrimidine biosynthesis pathways for survival. Interestingly, dicoumarol a complex I inhibitor, also blocks both respiratory chain function as well as pyrimidine biosynthesis in human myeloid leukemia HL-60 cells (Gonzalez-Aragon et al., 2007) and Gattermann et al. (2004) obtained similar results in both normal human keratinocytes and an osteosarcoma cell line. Thus, inhibitors of DHODH could contribute to ROS production that emanates from their effects on DHODH or the respiratory chain (reviewed in Lenaz, 2001). Nevertheless, DHODH has not proven to be a particularly effective target for the general therapy of cancers.

11. Concluding Remarks:

A prevailing and commonly held viewpoint is that glycolysis provides the major carbon source for tumor cells. However, the aim of this review is to consolidate and strengthen an opposing viewpoint that mitochondrial bioenergetic pathways maintain vital functions required for tumor cell growth and survival. Because of the depth of information in this area, evidence supporting the many alternative energy sources important for maintaining mitochondrial function and survival of tumor cells is emphasized using selected examples that highlight this aspect. However, it should be noted that the listed examples contained herein is by no means complete, as many other inhibitors of the respiratory chain complexes or other mitochondrial targets exist that are highly selective for cancer cell mitochondria and which induce ROS triggered apoptosis (reviewed in Neuzil et al., 2007; Ralph and Neuzil, 2009). This review also explores the potential of using these tumor mitochondria-linked bioenergetic pathways as targets for cancer therapy. Unlike oncogenes which mutate frequently in cancers leading to resistance to therapy, mitochondrial bioenergetic pathways may prove to be invariant targets with universal application as therapies that will work across all cancer types. Nevertheless, one of the major points to emerge

from the review is the substantial quantity of evidence that strongly supports an overlooked point made in Warburg's (1956) seminal publication that if the respiration falls below a certain minimum that the (cancer) cells need unconditionally, despite their increased glycolysis, they die. The substantial evidence provided here leads us to propose that the dependency on mitochondrial function in cancer cells exposes their vulnerability as an important drug target. In addition, another unifying theme that emerges from the divergent approaches used for molecularly targeting specific mitochondrial bioenergetic pathway differences in cancer *versus* normal cells is the importance of this targeting to trigger mitochondrial disruption leading to excessive ROS production, thereby promoting apoptosis (summarized in Table III). Although not absolutely essential for the activation of cell death, mitochondrial disruption with excess ROS production provides a very effective approach for cancer therapy selectively activating apoptosis in cancer cells. One of the best characterized examples of targeting mitochondria in cancer cells to activate ROS triggered apoptosis is that provided by the mitocans based on vitamin E analogues, which affect the UbQ binding sites on complex II, as outlined in section 2.4. These drugs have shown high potency at inhibiting tumor development in pre-clinical models. Preliminary evidence indicates that mitocans shall also complement existing therapies to enhance their activity against cancer.

As alternative energy sources, tumor mitochondria will oxidize a variety of FA (FAD-linked substrates) at similar or even higher rates than glutamine, pyruvate or malate (NAD-linked substrates) (see Table 1). On the contrary, KB oxidation by cancer mitochondria was lower compared to these other NAD- or FAD-dependent substrates. Many different amino acids, odd chain fatty acids and short carboxylates such as propionic acid that can be catabolised by tumor cells enter into one or either of these pathways, *e.g.* proline or hydroxyproline as glutamine. Hence, an abundance of alternative sources exists for tumor cell mitochondria to catabolize. The high mitochondrial electrical membrane potential generated by palmitoyl-carnitine (+ malate) also indicates the ability of tumor mitochondria to oxidize FA, in a process associated with H⁺ pumping

by the respiratory chain (data not shown). On the other hand, although analysis of acetoacetate did not clearly show a stimulation of State 3 respiration, high membrane potentials were generated (data not shown). These observations clearly indicate that FA and KB oxidation may be a metabolic strategy that tumor cells readily activate when responding to glucose and glutamine deprivation, and the review highlights how these other oxidative pathways could be alternative target sites for cancer treatment.

Unfortunately, novel therapeutic approaches targeting mitochondrial bioenergetic pathway differences in cancer cells are still in the early stages of development and as is often the case, more questions are raised than are answered. Perhaps the most pressing question now is how effective will these alternative therapies prove to be in treating cancers and will they survive the scrutiny of clinical trials in human cancer patients. An important test will be whether these treatments show longer term toxicities related to the greater sensitivity of cellular metabolism in the brain, similar to the many neurotoxicities associated with congenital methylmalonate and propionic acidurias. It also remains to determine which of the mitochondrial-linked bioenergetic pathways are the most critical for inducing different cancer cells to undergo apoptosis, or if any single pathway can be shown to be critical for the continued survival of all tumor cells. Consequently, it may be more effective to target several different pathways simultaneously. More specific questions also remain in relation to the most important regulatory steps in whichever critical pathways are determined biochemically to provide the best candidates for targeting. For example, whether glutaminase and/or the glutamine transporter constitute the true rate-controlling steps of glutamine oxidation in cancer cells or whether the flux controls reside in other pathway steps (*e.g.* malic enzyme or 2OGDH). In addition, in cancer cells where mitochondrial β -oxidation is highly active, determining whether CPT-I is the rate-limiting step must await further experimental evaluation. Unfortunately, the inhibition constant (K_i) values for malonyl-CoA on CPT-I activity in cancer cells have yet to be determined as these would help provide us with some answers. In addition, the

role of HMGCS 2 as a controlling step of ketogenesis should be addressed and whether all types of tumors exhibit significant down-regulation of HMGCS 2 or not.

Studies on tumor cells are also incomplete in that most analyses of glutamine, FA and KB oxidation pathways have been carried out under aerobic conditions and/or with cells cultured in aerobiosis. However, as is increasingly becoming apparent, many tumors show extensive regions of hypoxia. Therefore, more careful examination will be required into how glutaminase, proline or hydroxyproline oxidases, malic enzyme, glutamine transporters, CPT-I, HMGCS 2, and SCAAT expression are regulated under hypoxic conditions in cancer cells and the relationship to hypoxia-induced factors such as HIF-1. Furthermore, given the ability of invasive cancer cells to obtain proline and hydroxyproline from their extracellular matrix when glutamine, glutamate and glucose become deprived, another question is whether increased expression of POX and OHPOX will enable these amino acids to substitute as energy sources maintaining the formation of glutamate and metabolites such as lactate, ATP and the reducing equivalents necessary for cell growth and survival or whether another energy source such as short chain carboxylates like propionic acid will become dominant.

Finally, given the knowledge that caloric restriction and dietary intervention can significantly prolong life, it is highly likely that these life choices will lead to either no cancer or delays in its occurrence. In which case, what will be the long term benefits of interventions with AICAR, PUFA's and PPAR agonists (fibrates, glitazones) on the global incidence of cancer and will they provide us with immunity from conspicuous consumption, without side-effects? Hopefully, all of the above questions will be answered.

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Figure Legends



Figure 1. Glutaminolytic pathway () and pyruvate metabolism () in cancer cells (1) Extracellular glutamine is transported into cytosol by the ASCT glutamine transporter; (2) in the cytosol, glutamine is transported to the mitochondrial matrix by a glutamine specific mitochondrial transporter; (3) mitochondrial glutamine is substrate of an over-expressed Pi-dependent glutaminase; (4) glutamate is dehydrogenated by glutamate dehydrogenase to form 2-oxoglutarate which enters in the Krebs's cycle; (5) mitochondrial malate is transported to cytosol by an active aspartate-malate shuttle; (6) cytosolic malate is reversibly decarboxylated to pyruvate by an over-expressed NADP⁺-dependent malic enzyme rendering NADPH and CO₂; (7) pyruvate is oxidized to lactate by the lactate-dehydrogenase type A; (8) mitochondrial citrate is expelled to cytosol to feed lipids and cholesterol synthesis; (9) mitochondrial pyruvate is actively dehydrogenated by the PDH complex to form acetyl-CoA. Also, PDH complex can form acetoin by condensing an active acetaldehyde (Acetal) with a decarboxylated pyruvate (as hydroxyethyl-thiamine-pyrophosphate-[HE-TPP] enzyme complex); (10) NADH formed by Krebs cycle and (11) proline oxidation also feeds the respiratory chain complexes for ATP supply. Up arrows represent high activity and enzyme expression (steps 3, 6, 7) compared to non-tumorigenic cells. Abbreviations: Ala, alanine; Asp, aspartate; Cit, citrate; Gln, glutamine; Glu, glutamate; Lac, lactate; Mal, malate; Pyr, pyruvate; P5C, Δ-pyrroline-5-carboxylate; RC, respiratory complexes; Succ, succinate.

Figure 2. Fatty acids and ketone bodies oxidation in liver (A) and tumor or peripheral organ (B) mitochondria.

Up-arrows represent high activity and enzyme expression, whereas down-arrows represent lower activity and expression, compared to non-tumorigenic cells. Oxidation of butyrate might initiate through two different reactions, one catalyzed by activation with short-chain acyl-CoA synthetase in the cytosol, and another catalyzed by SCAAT in the mitochondrial matrix. The reaction specificity (acetoacetate *versus* butyrate) can be established with purified SCAAT. Abbreviations: Carn, carnitine; MTP, mitochondrial trimeric protein; HMGCS 2, 3-hydroxy-3-methylglutarylCoA synthase 2; HMGCoA, 3-hydroxy-3-methylglutarylCoA; D-β-OHbut DHase, D-β-hydroxybutyryl dehydrogenase; 3-OHbut, 3-hydroxybutyrate; SCAAT, succinylCoA-acetoacetyl transferase; ButCoA, butyrylCoA; UQ, ubiquinone; RC, respiratory chain; DHOrot, dihydroorotate; Orot, orotate; DHODH, dihydroorotate dehydrogenase..

Figure 3. Chemical structure of PPAR_γ natural and synthetic ligands

Figure 4. Propionate oxidation pathways.

The key steps involved in use of a range of amino acids, propionic acid and other metabolites that feed into propionyl-CoA and on to the Krebs' cycle in tumor cells are shown. The importance of malonyl-CoA, malonate and methylmalonate as potential inhibitors that could induce tumor mitochondrial disruption or succinate build up and pseudohypoxia are also shown.

Abbreviations: ACAC, Acetyl-CoA Carboxylase; CPTI, Carnitine palmitoyl transferase I; DC, Dicarboxylate carrier; FAS, Fatty Acid Synthase; MCAE, Malonyl-CoA epimerase; MMCAE, Methylmalonyl-CoA epimerase; MMCAM, Methylmalonyl-CoA mutase; MMSDH, Methylmalonate semialdehyde dehydrogenase; PCAC, Propionyl-CoA Carboxylase; PCAS, Propionyl-CoA Synthetase; SDH, Succinate Dehydrogenase. All enzymes indicated in blue are intra-mitochondrially located.

Table 1. Active free fatty acid, pyruvate and malate oxidation by hepatocarcinoma isolated mitochondria

SUBSTRATE	Pseudo STATE 4	STATE 3	STATE 4	Respiratory Control
none	22	10	10	1
ngAO/min/mg protein				
NAD⁺-LINKED SUBSTRATES				
0.1 mM Mal	30	32	26	1.2
10 mM Mal	53	103	119	1.1
5 mM Pyr + 0.1 mM Mal	55	228	50	4.5
5 mM Gln + 0.3 mM Mal	27	40	24	2
5 mM Proline+ 0.1 mM Mal	34	21	21	1
KETONE BODIES AND FREE FATTY ACIDS				
10 mM β -OHbutyrate + 0.1 mM Mal	29	34	34	1
5 mM AcAc + 0.1 mM Mal	21	30	27	1.2
0.5 mM Acetyl-DL-carnitine + 0.1 mM Mal	33	42	33	1.2
50 μ M Octanoyl-carnitine + 0.1 mM Mal	65	160	75	2.4
10 μ M Palmitoyl-carnitine + 0.1 mM Mal	67	96	51	1.8

Rat AS-30D mitochondria were isolated as described previously (Marín-Hernández et al., 2003). Mitochondrial respiration (1-3 mg protein) was assayed in 1.9 ml oxygen-saturated KME medium (120 mM KCl, 20 mM MOPS, 1 mM EGTA, pH 7.2) containing 5 mM phosphate, and the indicated oxidable substrate. Oxygen consumption was determined polarographically with a Clark-type electrode at 37°C. Mitochondrial state 3 was stimulated with 530-600 nmol ADP. Data shown represent the mean of at least two different preparations. Abbreviations: AcAc, acetoacetate; Gln, glutamine; Mal, malate; Pyr, pyruvate; PSD STATE 4, pseudo state 4; RC, respiratory control.

Table 2. Relevant enzyme activities of glutaminolysis pathway in tumor and normal mitochondria and cells

ENZYME	SOURCE	ACTIVITY	REFERENCE
Mitochondrial Phosphate-dependent glutaminase	Normal		
	Liver mitochondria	2.5-20 mU/ mg protein	Verhoeven et al., 1983; McGivan et al., 1980; Kovacevic et al., 1995. Rodríguez-Enríquez, Hernández-Esquivel, Moreno-Sánchez, unpublished data.
	Brain homogenate	28-63 mU/ mg protein	McGeer and McGeer, 1979
	Tumor		
	AS-30D Ehrlich ascites mitochondria	60-82 mU/ mg protein	Quesada et al.,1988; Rodríguez-Enríquez, Hernández-Esquivel, Moreno-Sánchez, unpublished data.
Mitochondrial Glutamate dehydrogenase	Normal		
	Liver mitochondria	1.4 U/mg	Rodríguez-Enríquez,, Hernández-Esquivel, Moreno-Sánchez , unpublished data.
	Brain and liver homogenates	5 U/mg	MacCarthy and Tipton,1984;
	Tumor		
	AS-30D and Ehrlich ascites mitochondria	0.73-0.75 U/mg	Moreadith and Lehninger, 1984; Rodríguez-Enríquez, Hernández-Esquivel, Moreno-Sánchez, unpublished data.
Cytosolic NADPH-dependent Malic enzyme	Normal		
	Liver, heart, insulin secreting INS 832/13 cellular extracts	5-20 mU/ mg protein	Zélewsky and Swierczynski, 1991; Kurz et al., 1993; Loeber et al., 1994; Guay et al., 2007
	Tumor		
	Human breast cancer, MCF-7 ,MDA-MB-453 breast cancer cellular extracts	19-37	Chang et al., 1991; Mazurek et al., 1997

Table 3. Summary table of bioenergetic pathway targets, selective drug inhibitor/activator and ROS involvement in mediating cancer cell death.

Review Section	Bioenergetic Pathway	Inhibitor/Activator	Role of ROS
2.3	Pyruvate/PDH/PDK	Lipoic acid	YES
		Dichloroacetate	YES
2.4	Complex II/SDH	α -TOS	YES
4.1, 4.4	Carnitine Palmitoyl Transferase CPT1/ □-oxidation of fatty acids	Etomoxir	YES
4.1	Dicarboxylate transporter and/or SDH in complex II	MethylMalonate Malonate and their CoA forms	YES
4.3	UCP2	Sulphur-substituted fatty acid analogues Tetracylthioacetic acid (TTA) and Wy14643	YES
5.8	Proline oxidase (POX) or hydroxyproline oxidase (OH-POX)	p53, PPAR γ	YES