Anti-cancer drugs that target metabolism, is dichloroacetate the new paradigm?

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Abbreviations DCA dichloroacetate, LDH lactate dehydrogenase, PDC pyruvate dehydrogenase complex, PDH pyruvate dehydrogenase, PDK pyruvate dehydrogenase kinase, PDP pyruvate dehydrogenase phosphatase, HIF1 hypoxia-inducible factor 1,

ABSTRACT

Recent findings in the fields of oncogenic regulation of metabolism, mitochondrial function and macromolecular synthesis have brought tumor metabolism and the Warburg effect back into the scientific limelight. A number of metabolic pathways that seem to be important for tumor growth are being touted as novel targets for anti-cancer drug development. One of the candidates in this class of drugs being investigated is dichloroacetate, a molecule used for over 25 years in the treatment of children with inborn errors in mitochondrial function. This pyruvate mimetic compound stimulates mitochondrial function by inhibiting the family of regulatory pyruvate dehydrogenase kinases (PDK1-4). The stimulation of mitochondrial function, at the expense of glycolysis, reverses the Warburg effect, and is thought to block the growth advantage of highly glycolytic tumors. Interestingly, some of the recent *in vitro* findings have shown very modest "anti-tumor cell activity" of DCA when cells are treated in a dish. However, several studies have reported "anti-tumor activity" in model tumors. This apparent paradox raises the question, how do we evaluate cancer drugs designed to target tumor metabolism? Traditional approaches in cancer drug development have used *in vitro* assays as a first pass to evaluate potential lead compounds. The fact that DCA has better *in vivo* activity than *in vitro* activity suggests that there are unique aspects of solid tumor growth and metabolism that are difficult to recapitulate *in vitro*, and may be important in determining the effectiveness of this class of drugs.

INTRODUCTION

In the past 20 years, the number of manuscripts containing "tumor metabolism" has increased from 3 to 28 per year, and the number of times these articles have been cited has increased from 23 to 929 per year (ISI statistics). The renewed interest in understanding the mechanisms and consequences of altered tumor metabolism has clearly captured the imagination of the scientific community. The idea that tumors have altered metabolism was first recognized by Nobel Prize-winning biochemist Otto Warburg when describing glucose metabolism[1]. More recently, the concept that tumors are metabolically different has grown to encompass other characteristics, such as glutaminolysis, fatty acid oxidation, and lipid biogenesis. There is clearly a different metabolic demand that drives these changes in cells that are continuously dividing when compared to terminally differentiated cells. The discovery of these alterations has raised the possibility that they may be therapeutically targeted due to their unique importance to cancer cells[2].

The concept that metabolic changes are a response to unique demands within the tumor has been proposed[3], even when it is hard to quantitate those demands. There is an interplay between oncogenic changes in the tumor cell with the unique aspects of the tumor microenvironment that impact on cellular metabolism and vice versa (Figure 1). It is therefore difficult to establish the exact metabolic demands within the tumor by studying the cells from the tumor grown *ex vivo*. The environmental conditions used to grow cells in culture are very different from the environmental conditions *in vivo*. High glucose Dulbecco's modified Eagles media (DMEM) and an atmosphere of 21% oxygen is very different from the hypoxic and/or hypoglycemic conditions found in the tumor[4-5]. The glucose concentration of 25 mM is approximately five times that of normal blood levels, and the oxygen tension is at least four times greater than that found *in vivo*. The fact that the cells are bathed in these metabolic substrates significantly alters their inherent metabolic programs[4, 6]. Elevated glucose concentrations favors glycolysis (the Crabtree effect[7]) while elevated oxygenation produces increased oxygen byproducts, and shortens cellular lifespan[8]. Glucose metabolism illustrates the interplay of these three factors in the tumor. Oncogenic transformation drives tumor cell proliferation in excess of vascular capacity, generating hypoxia. Hypoxia within the tumor microenvironment enhances glycolytic metabolism, largely through the activation of the HIF1 transcription factor[9]. Increased glycolysis leads to increased production of lactate, which contributes to an acidic extracellular pH and further changes in gene expression[10]. Both hypoxia and acidosis can contribute to increased levels of somatic mutation that can further drive tumor progression[11-12]. It is clearly difficult to reproduce these complex interactions in cells grown *in vitro*.

Part of the interaction between the microenvironment and tumor cell metabolism is generated through an adaptive response to dynamic changes in cellular supply and demand for metabolites. The simple fact that we can measure regions of hypoxia and acidosis within tumors indicates that the tumor vessels do not maintain a constant environment for the growth of the tumor cells[13]. The tumor vasculature represents a bottleneck in the delivery of nutrients and the removal of waste products from the tumor [4]. The inadequate supply from the tumor vessels initiates an adaptive response from the tumor cells designed to decrease the demand for the limited metabolites. This dynamic process is difficult to model *in vitro* (Figure 2). For example, low levels of oxygen within the tumor induce HIF1 transcription factor

and its metabolic program[9]. Part of the HIF1-initiated metabolic program is to reduce oxygen demand by decreasing mitochondrial function. Part of this response is mediated through the HIF1-dependent induction of PDK1 and PDK3 within the tumor cells, and a reduction in pyruvate oxidation within the mitochondria[14-17]. This adaptive response is responsible for bringing the demand for oxygen closer to the limited supply.

Dichloroacetate (DCA) is capable of interfering with this adaptation to tumor hypoxia by inhibiting function of the PDKs (Figure 3). The block of an adaptive response to hypoxia is most clearly observed when the tumor as a whole is in oxygen deficit, and the tumor vasculature cannot respond to this increased demand[18]. Even when tumor cells are placed in hypoxia, there is enough oxygen in the environment (1-2%) to maintain a stable, albeit low oxygen condition within the cell even with the addition of DCA. The rate of diffusion into the cells is faster than the rate of consumption, so the intracellular level of oxygen does not depend on the rate of consumption (unless very high cell numbers are used in glass dishes that prevent diffusion of oxygen through the plastic[14]). In this manuscript, we present an analysis of the published data regarding DCA as an example of a drug designed to impact tumor metabolism that supports our hypothesis that this class of drugs has a very different effect on tumor cells growing *in vivo* with the vasculature-produced metabolic bottleneck, when compared to their effect on cells grown *in vitro*.

THE PYRUVATE DEHYDROGENASE COMPLEX (PDC)

Inhibition of mitochondrial function is as important as increased glycolysis to produce the Warburg effect. One major regulator of mitochondrial function is the pyruvate dehydrogenase complex (PDC), which catalyzes the irreversible decarboxylation of pyruvate to acetyl-CoA, CO_2 and NADH. Controlling PDC controls the entry of carbons derived from carbohydrates into the mitochondria. This reaction plays a central role in regulating both mitochondrial energy producing pathways (TCA cycle and OXPHOS) and the generation of biosynthetic intermediates, such as citrate. PDC consists of three catalytic components, pyruvate dehydrogenase (E1), dihydrolipoamide transacetylase (E2) and dihydrolipoamide dehydrogenase (E3), which are organized into large multimeric complexes together with the structural subunit E3 binding protein (E3BP). The basic core of the E1 pyruvate dehydrogenase component is a heterotetramer of two alpha and two beta subunits ($\alpha_2\beta_2$), and it catalyzes the first step of pyruvate decarboxylation. The activity of the complex is largely regulated by the reversible phosphorylation of three serine residues of E1α. Pyruvate dehydrogenase kinases (PDK1-4) inactivate PDC and pyruvate dehydrogenase phosphatases (PDP1-2) activate/reactivate it (Figure 3).

The different PDK isoforms vary in their regulatory properties, tissue distribution and modulation by upstream metabolic signals. This results in dynamic, organ-specific control of mitochondrial function and energy production. Enzymatic assays have shown that the PDKs differ in their specificity towards the three E1α target sites and the kinetic parameters of phosphorylation. PDK1 is the only isoform able to phosphorylate all three sites, whereas PDK2-4 phosphorylate sites 1 and 2 with different rates *in vitro*[19-20]. Phosphorylation of even one of the six E1α sites in the heterotetramer is sufficient to inactivate PDC and it is believed that maximally three out of the possible six sites of the E1 tetramer can be

phosphorylated any given time[21-22]. Spatiotemporal changes in the levels and activity of the PDC phosphatases (PDP1-2) are also regulatory. Dephosphorylation *in vitro* appears to be random; both isozymes are able to dephosphorylate the three E1 sites of recombinant mutated substrates[23].

DICHLOROACETATE: A PDK INHIBITOR

Dichloroacetate was identified as a pyruvate dehydrogenase activator through its ability to stimulate PDC enzymatic activity in a perfused rat heart model[24]. It is now known that this pyruvate mimetic acts by inhibiting the action of the PDKs. The crystal structure of PDK2 in complex with DCA has been obtained, and it shows that DCA occupies the pyruvate binding site in the N-terminal regulatory (R) domain[25]. The four isozymes vary considerably in their sensitivity to inhibition by DCA. PDK2 is the most sensitive, PDK3 the most resistant, while PDK1 and PDK4 are relatively sensitive[26-27].

Treatment of lactic acidosis with DCA

The safety and efficacy of DCA has been evaluated in cases of congenital and acquired lactic acidosis. DCA treatment effectively reduces lactate levels in the circulation by stimulating oxidation of pyruvate, however, it is still not known if DCA can improve the prognosis of patients with these syndromes[28-29]. It has been proposed that young children with PDH deficiency may benefit the most from chronic DCA treatment[30].The most significant adverse effect of long term DCA administration is a reversible peripheral neuropathy[31-32]. The severity of the toxicity appears to be age-dependent, with adult patients being more susceptible than children[31, 33]. The reasons for this discrepancy are not entirely clear, but they are possibly related to the different pharmacokinetics and metabolism of DCA in the two age groups[34]. DCA has also been used in clinical trials for heart disease, including congestive heart failure and ischemic heart disease, showing positive results and improving myocardial performance[35-36]

DCA as a potential cancer therapeutic

In recent years, DCA has attracted attention as a potentially simple and economical means to target glycolytic tumors while producing limited side effects in the oxidative healthy organs. The interest in this drug by the scientific community, cancer patients and the media was kindled in 2007, after a group from the University of Alberta reported that DCA was uniquely toxic to human cancer cell lines and inhibited the growth of A549 lung tumor xenograft tumors in rats[37]. Since then, the emerging reports on the efficacy of DCA *in vitro* and *in vivo* reveal some interesting and also puzzling characteristics, which distinguish the case of DCA from the majority of drugs developed as anti-cancer agents (table 1). The number of different cancer types and experimental strategies tested to-date is too limited to allow for generalized conclusions about the efficacy of DCA against all kinds of tumors. With this caveat, a qualitative comparison of the literature suggests that DCA shows more anti-cancer effect *in vivo* than anti-cancer cell effect *in vitro*.

***In vitro* studies**

DCA has been reported to have cytotoxic effects *in vitro*[37-40], with some responses at clinically relevant concentrations (0.5-1 mM), while others require supra-pharmacologic levels (10-100 mM), and still other groups have found no direct toxicity *in vitro*[18, 41-43]. One condition that has been identified that sensitizes cells to DCA is mutations that perturb mitochondrial respiratory function[42-43], suggesting that enforced utilization of defective OXPHOS is toxic. Since oxygen deprivation also downregulates mitochondrial function, it seemed reasonable to hypothesize that hypoxic cells would be more sensitive to DCA. However, this hypothesis has not been supported, at least in the limited number of cell lines tested to-date. Moderate *in vitro* hypoxia did not influence the cell cycle profile of DCA-treated colorectal cells[44] or the reproductive viability of DCA-treated pancreatic cancer cells (our unpublished observations). Interestingly, another study found severe hypoxia (anoxia?) can be protective against DCA-induced apoptosis in colorectal cancer cells[45]. The reason for these discrepancies is not clear.

Overall, the majority of the data support the idea that clinically relevant concentrations of DCA (less than 1 mM) are not directly cytotoxic *in vitro*. The reason for this apparent cellular resistance is not an inactivation of DCA under tissue culture conditions or an inability to inactivate PDKs, since DCA has been shown to transiently increase mitochondrial activity and collapse the mitochondrial membrane potential[18, 37, 46]. Therefore, the basis for the limited anti-cancer effect of DCA in culture likely lies in the complex cellular physiology and the enormous excess of metabolites present in culture media.

Pre-clinical models

Reports of DCA activity against model tumors grown in rodents are encouraging, although there are certainly cases of tumor lines that do not respond to treatment, and even one example of accelerated tumor growth in response to DCA[45]. The first report of DCA's anti-tumor activity was that of Bonnet and collegues[37]. The authors reported that A549 lung adenocarcinoma xenografts grown in nude rats showed significant tumor growth delay after treatment with DCA, with some experimental groups even showing tumor regression. These effects were associated with increased apoptosis and reduced proliferation. Using the same A549 cells, Stockwin et al, recently confirmed that DCA was growth-suppressive in model tumors, even though they found little toxicity *in vitro*[42].

Our group has reported that daily DCA treatment of mice with pancreatic SU86.86 xenografts caused a significant tumor growth delay, as well as an increased hypoxic fraction of the tumors. We hypothesized that increased mitochondrial oxygen consumption resulted in greater hypoxia which was growth inhibitory to the tumor[17]. In support of this model, DCA increased the extent of tumor hypoxia in RKO colorectal xenografts as assessed by either HRE-driven luciferase reporters[47] or ^{18}F-fluoroazomycin arabinoside positron emission tomography[18]. This RKO model showed very modest reduction in the growth of the DCA-treated tumors, but the acute changes in oxygenation post DCA sensitized them to treatment with hypoxia-activated cytotoxins such as Tirapazamine[47] or PR-104[18]. Additional work on colorectal cancer models, by other groups, has revealed significant heterogeneity in the response to DCA. Some cell lines reduce their tumor growth rates[48], whereas others either failed to respond or even

grew faster[45]. Additionally, in a rat model of mammary adenocarcinoma, an intensive schedule of DCA treatment was able to reduce the number of macroscopic lung metastases[49]. These pre-clinical models support the concept that DCA has the ability to modulate tumor metabolism *in vivo*, resulting in greater of lesser anti-tumor effects based on the model tested.

Clinical trial data

The first data on the evaluation of DCA for the treatment of human cancer were reported recently[50]. In this study, DCA was used in combination with surgery, temozolomide (TMZ) and radiation for the treatment of 5 patients with glioblastoma multiforme (GBM). While the authors report promising clinical results in 4 of the 5 patients, the emphasis of the report was the *ex vivo* analysis of the tumor cells before and after treatment with DCA. They report changes in mitochondrial membrane potential, increased amounts of mitochondrially generated oxygen radicals, and increased tumor cell apoptosis. Mechanistic studies found altered levels of HIF1 signaling, p53 activation and decreased angiogenesis. These data suggest that DCA has many mechanisms of action downstream of its inhibition of PDKs. Obviously greater numbers of patients, preferably from a number of different sites, need to be treated to be able to generalize these exciting results.

ORGANISMAL REGULATION OF METABOLISM

Humoral growth- and nutritional factors could also influence tumor response to metabolic reprogramming. The regulation of tumor PDC activity *in vivo* has not been systematically studied yet, but the knowledge we have gained from normal tissue metabolism and the fields of endocrinology and obesity research provide some interesting hypotheses. Starvation increases the expression of PDKs and decreases PDH activity in peripheral organs as a strategy to maintain a stable supply of carbohydrates to the brain and other neuronal tissues. For example, starvation transcriptionally activates PDK4 and PDK2 in liver, kidney and other tissues[51-52]. Glucocorticoids, T3 thyroid hormone and free fatty acids also increase PDK4 levels[53]. Re-feeding and/or increases in insulin levels decrease PDK4 transcription and reactivate PDC. Likewise, starvation and diabetes were shown to downregulate PDP2 mRNA and protein levels, effects that were reversed by re-feeding or treatment with insulin[54].

Therefore, the many input signals that impact PDH activity *in vivo* could influence the efficacy of DCA and other metabolically-targeted drugs. Delivery of the drug during fasting could have a very different effect when compared to the fed state. The responses to humoral conditions are obviously different for different tissues and tumor types and so, difficult to mimic *in vitro*. So, is it possible to evaluate the anti-tumor effect of molecules like DCA *in vitro*? Many years of study of a large number of tumor cell lines grown in culture shows that they do mantain the abnormal characteristics of aerobic glycolysis in vitro, and therefore provide a valuable tool in the study of cancer metabolism[55]. However, there also inherent limitations in these systems, as evidenced by the inconsistent data in the literature when comparing the effect of DCA in cell culture versus preclinical and clinical outcomes.

The preceeding analysis presents the model that an anti-cancer drug that targets metabolism and has little toxicity *in vitro* may have significant potential *in vivo*. We have highlighted the limited metabolic substrates in the tumor, and the systemic regulation of metabolism by humoral factors that could increase *in vivo* drug efficacy. It is possible that the converse may also be true that a drug with good activity *in vitro* may have little success *in vivo*. Effects on normal tissue toxicity, or metabolic cooperation between cell types could limit a drug's effectiveness *in vivo*. The widely-tested metabolic inhibitor 2-deoxyglucose has reasonable anti-cancer activity *in vitro*, but cannot be used in patients due to its negative effects on normal tissues that rely on glucose consumption. The dose-limiting neurologic toxicity occurs at a drug level well below that needed for anti-cancer effects in rodent tumors[56-57]. Alternatively, it is possible that metabolic cooperation between cell types or normal and tumor cells may bypass the drug-induced metabolic block. For example, while lactate is often viewed as a metabolic waste product, it can be used in some cells as a fuel to power mitochondrial function[58-59].

With respect to combining DCA with existing therapies, the preclinical data so far do not show an obvious pattern of interaction that would allow for an easy and rational selection of therapeutic regiments. Animal models will continue to be the best means of testing to determine empirically the most promising combinations. We have shown that due to its ability to increase oxygen consumption, DCA increased tumor hypoxia and sensitized xenografted pancreatic and colon tumors to hypoxic cytotoxins[18, 47], so it is intriguing to envision a treatment plan including these two classes of drugs that are both designed to exploit the unique hypoxic microenvironment of the tumor.

The interaction of DCA with other metabolic modulators has not been reported. A potential target for combination therapy is lactate dehydrogenase A (LDH A). Genetic or pharmacologic inhibition of LDHA has been shown to increase mitochondrial function and inhibit model tumors' formation and progression[60-61]. In this combination scheme, the LDHA inhibitor would block the conversion of pyruvate to lactate and DCA would divert the accumulated pyruvate towards mitochondrial oxidation. If DCA's anti-tumor effect is derived from increased mitochondrial function, it is possible that combining PDK and LDHA inhibitors would force an even greater rate of mitochondrial oxidation, and impair tumor growth more efficiently.

Nodal points of key survival pathways, such as the PI3K-Akt-mTOR pathway are also the subject of intense drug development efforts[62]. Part of the growth promoting properties of this pathway comes from its ability to regulate metabolism and energy production by direct or indirect mechanisms. For example, oncogenic activation of PI3K-Akt stimulates glucose uptake and aerobic glycolysis[63-64], whereas activation of Akt and mTORC1 increases the translation of Hif-1α mRNA under hypoxia[65]. Promising Akt inhibitors and new generation mTOR inhibitors are being tested in clinical trials[66-67] and represent viable candidates for combination therapy with DCA to modulate both aerobic and hypoxic metabolism.

CONCLUSIONS

In conclusion, recent understanding about the unique metabolism of the solid tumor has identified several novel, druggable pathways that may be preferentially used in tumor cells compared to normal cells. Analysis of candidate anti-cancer drugs designed to target these metabolic pathways will require

careful experimental design, both *in vitro* and *in vivo*. Analysis of the published reports studying dichloroacetate shows a confusing, and sometime contradictory, range of *in vitro* and *in vivo* effects. Genetic studies in model tumors offer compelling evidence that this pathway is a good candidate for therapeutic targeting[68]. It would be very helpful in the analysis of the potential utility of DCA if there could be some molecular signature that could predict for drug senstitivity, both in model tumors and eventually in patients. Perhaps a careful analysis of the presumed target of DCA, the phosphorylation of pyruvate dehydrogenase E1α subunit may offer this signature.

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Cancer Type	Reference	Effect on survival and growth
In vitro studies		
Lung, Glioblastoma, Breast	Bonnet et al, 2007	Apoptosis *in vitro*, xenograft growth inhibition
Prostate	Cao et al, 2008	Growth inhibition *in vitro*, moderate radiosensitization
Endometrial	Wong et al, 2008	Growth inhibition
Cervical	Anderson et al, 2009	Growth advantage under hypoxia *in vitro*
Head and Neck	Sun et al, 2009	*In vitro* growth inhibition only in mutND2 overexpressing cells
Pediatric	Heshe et al, 2010	Apoptosis at high concentrations *in vitro*, some influence on response to chemotherapy
Colorectal	Madhok et al, 2010	Apoptosis at very high concentrations
Pre-clinical models		
Colorectal	Cairns et al, 2007	Little effect on growth, increased hypoxia by HRE-luciferase, sensitized to hypoxic cytotoxins
Colorectal	Cairns et al, 2009	Little effect on growth, increased hypoxia by 18F-FAZA PET, sensitized to hypoxic cytotoxin
Pancreatic	Chen et al, 2009	Xenograft growth inhibition
Colorectal	Sanchez-Arago et al, 2010	Xenograft growth inhibition
Colorectal	Shahrazad et al, 2010	Protected from anoxia *in vitro*, promoted xenograft growth of SW480
Colorectal, Breast, PML, Prostate	Stockwin et al, 2010	Active only against cells with defective electron transport chain
Breast	Sun et al, 2010	Inhibition of xenograft growth and metastasis
Human patient data		
Glioblastoma	Michelakis et al, 2010	Clinically stable disease *in vivo*, decreased HIF1, increased p53 ex vivo.

Table 1: Summary of published *in vitro*, pre-clinical, and clinical studies evaluating the anti-cancer effects of DCA. Some studies have both *in vitro* and model tumors, such as Bonnet et al 2007.

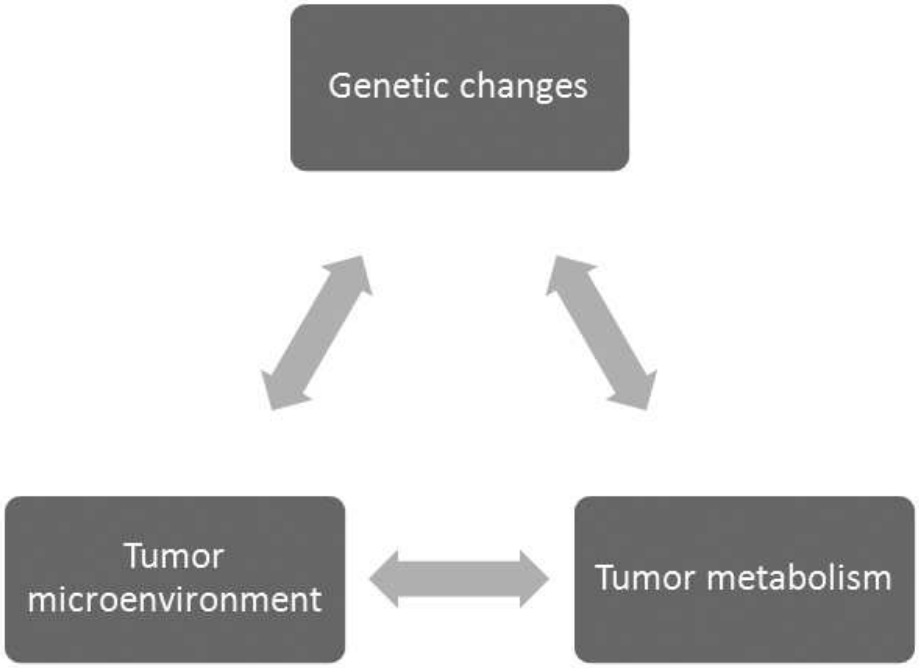

Figure 1 Interaction between oncogenic changes in tumor cells, with unique availability of metabolites in the tumor microenvironment, with the metabolic demand and adaptive capacity of the tumor cells. Note that all three factors influence each other and vice versa.
54x36mm (300 x 300 DPI)

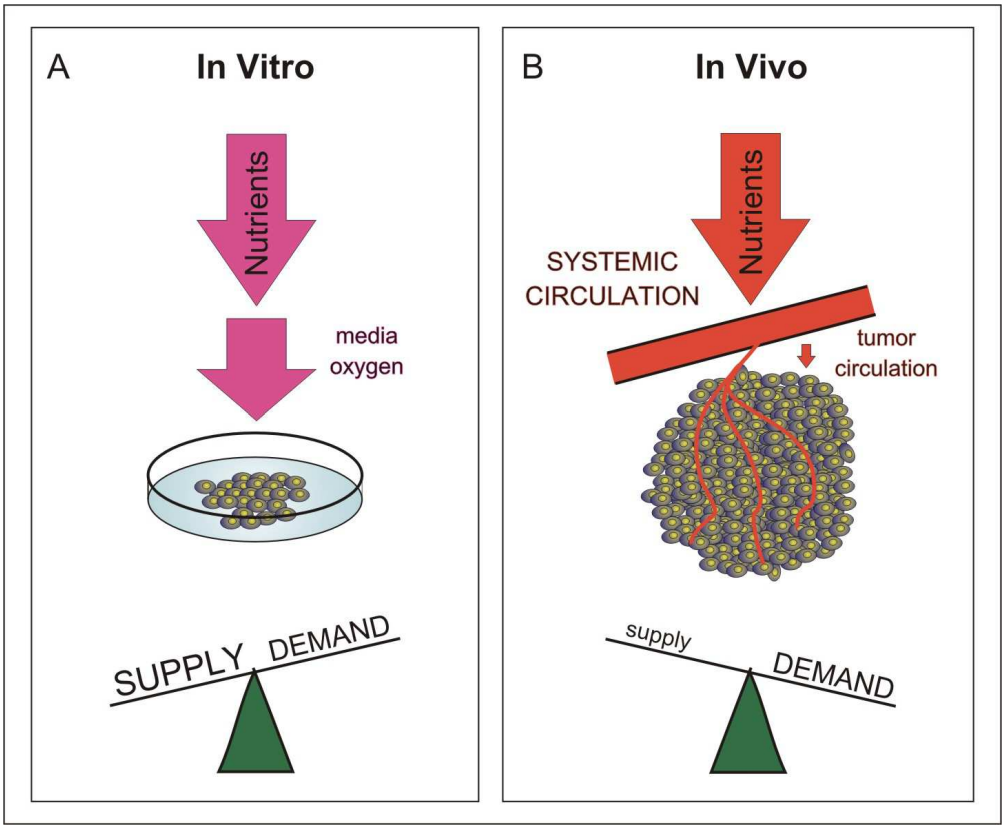

Figure 2 Concept of the vascular bottleneck limiting supply of metabolites to the tumor cells. Panel A describes in vitro conditions where there is a nearly unlimited supply of metabolites and oxygen such that consumption does not influence intracellular concentrations, and supply is always greater than demand. Panel B describes the in vivo condition where the tumor is fed by an inadequate tumor vasculature. The tumor is always in metabolic deficit with supply insufficient to meet demans. Subtle changes in metabolic demand in tumors can significantly affect overall levels of limiting metabolites.
122x100mm (300 x 300 DPI)

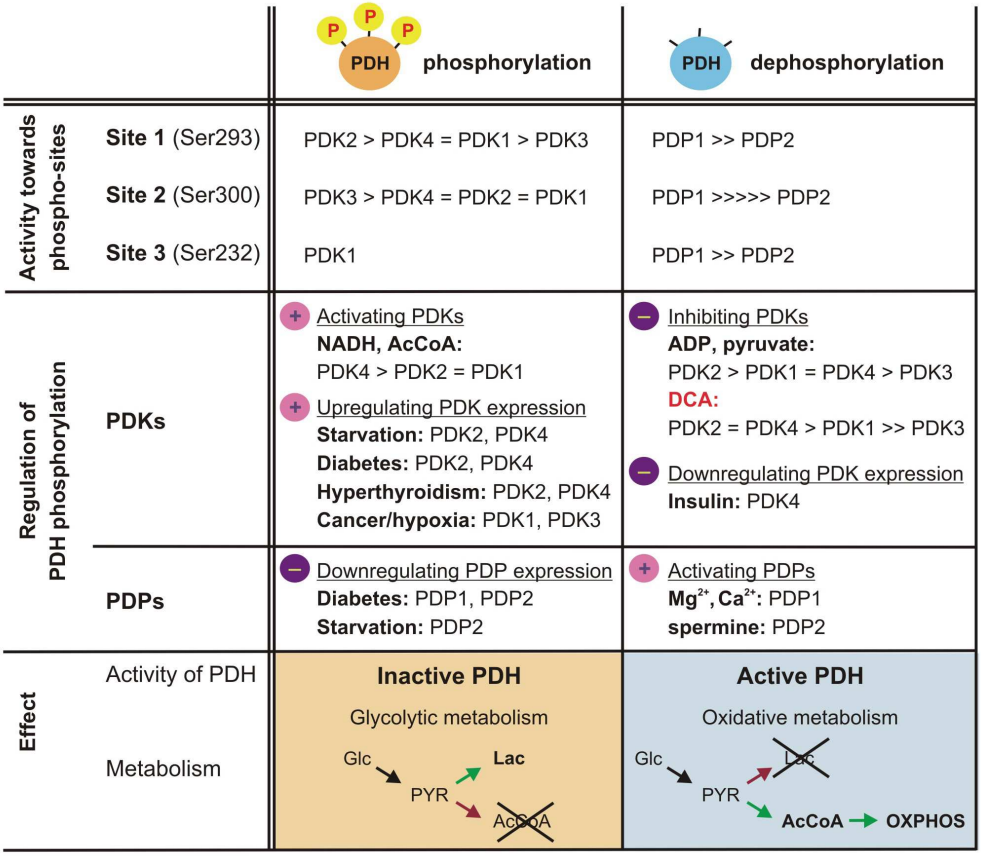

Figure 3 Regulation of pyruvate dehydrogenase activity by metabolites, pyruvate dehydrogenase kinases (PDKs), pyruvate dehydrogenase phosphatases (PDPs), and dichloroacetate (DCA). Consequences of these changes on cellular metabolism is shown at the bottom, with overall metabolism shifting from glycolytic to oxidative based on PDH activity.
171x149mm (300 x 300 DPI)