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Review

Metabolic effects of signal transduction inhibition in cancer assessed by magnetic resonance spectroscopy

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ABSTRACT

Despite huge efforts in development of drugs targeting oncogenic signalling, the number of such drugs entering clinical practice to date remains limited. Rational use of biomarkers for drug candidate selection and early monitoring of response to therapy may accelerate this process. Magnetic resonance spectroscopy (MRS) can be used to assess metabolic effects of drug treatment both *in vivo* and *in vitro*, and technological advances are continuously increasing the utility of this non-invasive method. In this review, we summarise the use of MRS for monitoring the effect of targeted anticancer drugs, and discuss the potential role of MRS in the context of personalised cancer treatment.

1. Introduction

The current understanding of cancer aetiology is that the human genome contains specific genes that are potentially oncogenic. Genetic events changing the function of these oncogenes may lead to malignant transformation. In some cases, such as chronic myelogenic leukaemia (CML), a single translocation may be sufficient for development of clinical disease (Quintas-Cardama and Cortes, 2009). More commonly, accumulation of several genetic abnormalities is needed for complete malignant transformation (Vogelstein and Kinzler, 2004). These abnormalities include both gain-of-function alterations in oncogenes, loss-of-function mutations in tumour suppressor genes and defects in genes involved in maintenance of genetic stability. The simultaneous occurrence of these mutations in single cells is statistically incompatible with the clinical incidence of cancer (Loeb et al., 2003). This suggests that the genotype of cancer cells is acquired in a step-wise manner, and that the cumulative effects of serially occurring genetic events finally enables cancer cells to proliferate autonomously, evade cell death and invade neighbouring tissue.

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Oncogenes are typically involved in signal transduction cascades related to growth, division and survival of cells, features which are important in normal development and differentiation of the organism. Following malignant transformation, these signalling cascades are perturbed and may stimulate abnormal cellular activity. Although a large number of oncogenes have been identified, many of them are involved in the same oncogenic signalling cascades. Therefore, different cancers have several properties in common, including metabolic abnormalities. The metabolic profile of cancer cells is determined in part by the genotype and in part by the tumour microenvironment. The mutual importance of these factors is not completely understood. Solid tumours typically have a restricted blood supply, leading to upregulation of response mechanisms which allow cancer cells to keep growing despite relative lack of oxygen and nutritients. However, the same response mechanisms may also be upregulated in leukaemic cells, suggesting that the genetic background of cancer cells is strongly associated with the metabolic phenotype.

The genetic abnormalities give cancer cells advantages in survival and proliferative capacity compared with their normal counterparts. This has lead to a new paradigm in anticancer drug discovery. The dysregulated signal transduction pathways in cancer contain a large number of potential drug targets, and drugs specifically targeting oncoproteins are continuously entering clinical practice. As shown by imatinib and similar drugs targeting the BCR-ABL fusion protein in CML, molecularly targeted anticancer drugs can have significant impact on clinical disease management (Leitner et al., 2010). Drugs specifically targeting dysregulated oncogenic signalling pathways are likely to have moderate effects on normal cells. In addition, such drugs allow individualisation of treatment, as the mutational profile of cancer cells can (and should!) be taken into account before selection of a therapeutic strategy.

Taking the drug development pipeline into account, the number of drugs targeting oncogenic signalling is likely to increase significantly in the near future. However, in order to accelerate the clinical development of molecularly targeted anticancer drugs, several obstacles have to be overcome. Due to extensive cross-talk between signalling pathways, the effect of blocking a single pathway may be difficult to predict. At the time of diagnosis, most cancer patients harbour functional mutations in several oncogenes, which further complicate rational use of targeted therapeutics. In contrast to classical chemotherapeutics, drugs targeting pathways regulating cell growth and proliferation often have a cytostatic (not cytotoxic) mode of action. Therefore, volumetric measurements alone may be inadequate for assessment of response to treatment. Indeed, development of functional biomarkers for treatment response may be one of the greatest challenges on the way towards personalised cancer management using targeted drugs (Myers and Cantley, 2010). Direct measurement of target activity would be the preferred approach, but to date this means use of invasive tissue sampling. Therefore, validation of surrogate biomarkers allowing non-invasive, repeated assessment of response to targeted drugs would be useful for drug discovery and development as well as clinical cancer management.

Among the imaging modalities which can be used to monitor functional changes in tumours is magnetic resonance spectroscopy (MRS), which reports on the concentration of various metabolites in cancer both *in vitro*, *ex vivo* and *in vivo* (Gillies and Morse, 2005). MRS may be particularly well suited for use with targeted drugs as some major metabolic pathways are regulated by oncogenic signalling pathways. This review addresses the use of various MRS methodologies for assessment of changes in cancer metabolism caused by molecularly targeted anticancer drugs.

2. Principles of magnetic resonance spectroscopy

Charged particles such as atomic nuclei have a magnetic property known as “spin”, allowing them to interact with a static magnetic field. At thermal equilibrium, nuclear magnetisation aligns along the axis of the magnetic field. By careful application of a radiofrequency pulse, which is chosen based on the particular nucleus and the magnetic field strength, the magnetic moment will (a) realign with the resultant magnetic field and (b) reach phase coherence. At this stage, the magnetisation rotates with a frequency proportional to the magnetic field strength. Different nuclei experience slight differences in the local magnetic field and resonate at slightly different frequencies. Following the excitation pulse, the magnetisation will return to thermal equilibrium through a process called relaxation, inducing an electric current in a receiver. Separation of the different resonance frequencies can be achieved by mathematically resolving the received signal. The signal strength is proportional to the number of nuclei resonating at each particular frequency and hence the concentration of chemical entities. Different chemical compounds have characteristic resonance frequencies termed their “chemical shift”. The chemical shift, together with coupling phenomena between neighbouring nuclei, allows identification and quantification of distinct chemical entities in the resulting spectrum of resonance frequencies. As the resonance frequency depends on the strength of the static magnetic field, both spectral resolution and signal intensity increases with magnetic field strength. The field strength of a magnet depends on its intended use. Clinical MR scanners typically operate at 1.5T or 3T, whereas narrow-bore research scanners for preclinical experiments operate at 7T or 11.7T. Spectrometers for analyses of biopsies or tissue extracts/biofluids operate at field strengths up to 23.5T (most commonly 11.7T or 14.1T). Equipment for *in vitro* and *ex vivo* MRS is tailored for analysis of small biological specimens, with high magnetic field homogeneity and transmit/receiver coils in close proximity to the samples. When MRS of biopsies, cultured cells or extracts is performed, the acquired signal represents the chemical composition of the entire sample. For MRS *in vivo*, it is crucial to measure the metabolic content in a well-defined region (i.e. a tumour) of the study subject. This is performed through the use of radiofrequency pulses allowing selective excitation of tissue and spatial localisation of the received signal. This can be done either in one single volume of interest or in a matrix of voxels covering the region of interest, a method often termed magnetic resonance spectroscopic imaging (MRSI). Spectra from biological specimens can be obtained with better sensitivity and spectral resolution *in vitro* and *ex vivo* compared to *in vivo*. This is illustrated in Figure 1 and

is caused by several factors such as higher field strength, better sample and magnetic field homogeneity, sensitivity of coils and the acquisition method used.

Although heavily relying on inherent quantum mechanical properties of nuclei, MRS has evolved into a highly flexible biochemical tool. Using MRS, metabolites containing nuclei such as ^{1}H, ^{19}F, ^{13}C and ^{31}P can be assayed. The advantages and limitations of different MRS methodologies are presented in Tables 1 and 2.

MRS does not discriminate between endogenous metabolites and exogenously administered substances. The metabolic fate of injected tracers can be examined based on different chemical shifts in the tracer and its downstream metabolites, allowing dynamic studies of metabolic flux in experimental systems. The use of ^{13}C-enriched tracers is the most common approach for this purpose, but other nuclei such as ^{19}F may also be employed. As the sensitivity of ^{13}C MRS is low, the use of ^{13}C-enriched tracers *in vivo* requires

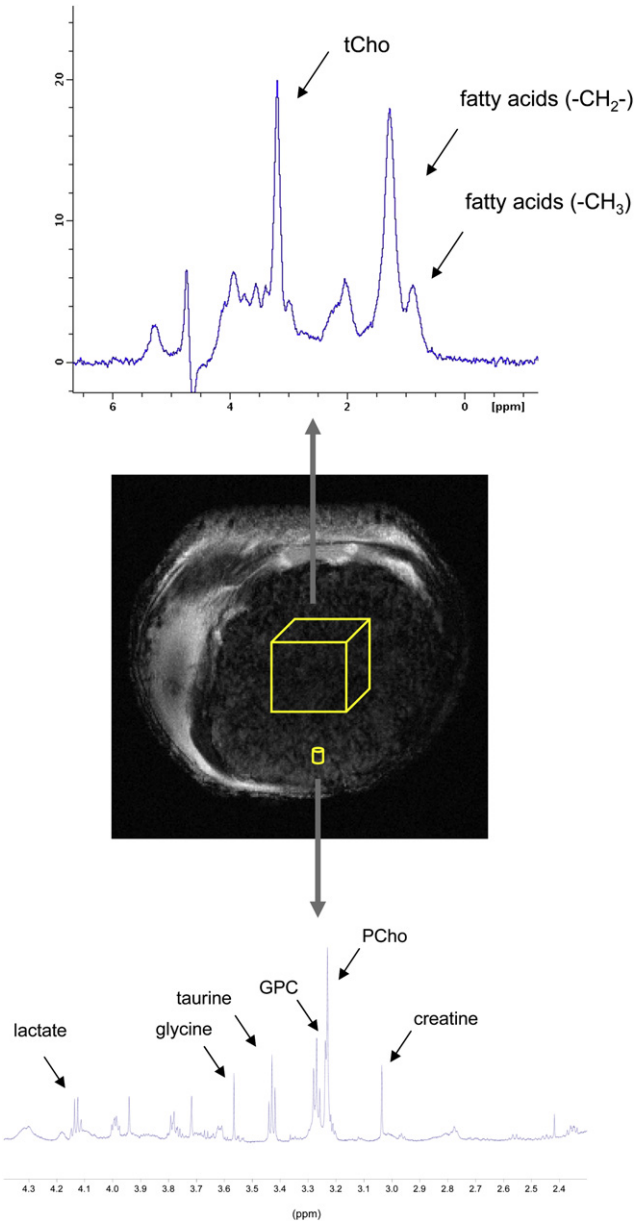

Figure 1 – Differences in sensitivity and spectral resolution between *in vivo* and *ex vivo* MRS. Following the acquisition of an anatomic MR image (middle), a volume of interest for spectroscopic assessment can be defined within the tumour (represented by the yellow voxel). The *in vivo* MR spectre (top) represents the signal obtained from this voxel. Harvesting a biopsy (represented by the yellow cylinder) from this tumour allows acquisition of an *ex vivo* spectrum at higher field strength (bottom). Image and spectra were obtained in a xenograft model of luminal-like breast cancer (MAS98.06) by the authors (unpublished data). The *in vivo* spectrum was obtained in a 7T small animal scanner (PRESS sequence, voxel size: 3 × 3 × 3 mm, scan time: ca 6 min). The *ex vivo* spectrum was obtained from a 15-mg biopsy from the same tumour, analysed using HR MAS MRS on a 14.1T spectrometer (CPMG sequence, scan time: ca 6 min). Some typical metabolites are identified in both spectra. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1 – Overview of advantages and limitations of various MRS applications.

Methodology	Advantage	Limitation
In vivo MRS	Allows measurement of metabolite content in tumours *in situ* using standard MR instrumentation. Similar methodology in preclinical and clinical applications facilitates translational studies. Non-invasive. Longitudinal studies possible. Spatial information available using magnetic resonance spectroscopic imaging (MRSI).	Signal broadening due to magnetic field inhomogeneity. Sensitivity (millimolar range) Quantification difficult. 1H MRS allows determination of total choline signal (tCho), but not individual choline metabolites.
Ex vivo MRS	By use of high resolution magic angle spinning (HR MAS), high spectral resolution can be obtained with minimal sample preparation Intact tissue specimens can be directly studied Intact cell suspensions can be studied directly Quantification possible Non-destructive method, sample available for further experiments	Invasive Longitudinal studies limited by the need for tissue samples Sample degradation may occur during analysis Sensitivity (micromolar range)
In vitro MRS	High spectral resolution. High sensitivity. Quantification through internal standards or other methods. Allows direct studies of perfused cell cultures No degradation of sample after extraction allows long acquisition time.	Invasive. Extraction of metabolites from tissue samples may influence the measured metabolite concentrations. Microenvironmental factors not taken into account. Sensitivity (low-micromolar)

administration of large amounts of tracer. However, the emergence of techniques for hyperpolarisation of injectable ^{13}C-enriched tracers has opened up new possibilities for real-time metabolic imaging *in vivo* (Golman et al., 2003). The signal intensity of MRS is limited by the low spin polarisation levels at thermal equilibrium (typically in the order of 0.0001% depending on nucleus and field strength). Through transfer of polarisation from electrons to nuclei at very low temperature in the solid state, the polarisation level can be increased to 25% or more, which represent a dramatic increase in MRS

Table 2 – Nuclei used in biomedical MRS and some typical applications in cancer.

Nucleus	Sensitivity	Typical applications and biomarkers in cancer
Hydrogen (^{1}H)	High sensitivity of detection due to high gyromagnetic ratio, high natural abundance and high concentration in tissue.	The most frequently used nucleus in *in vivo* MRS. Allows studies of choline, phosphocholine (PCho) and glycerophosphocholine (GPC). Other biomarkers in cancer research include N-acetyl aspartate, lactate, citrate, amino acids and lipids (Gillies and Morse, 2005).
Carbon (^{13}C)	Low sensitivity of detection due to low gyromagnetic ratio and low natural abundance. High spectral resoulution. High sensitivity can be obtained using exogenous tracers.	*In vivo*, *ex vivo* and *in vitro* studies of glucose and its downstream metabolites after administration of [1-^{13}C] glucose. Various endogenous tracers may be used for studies of other metabolic pathways (Gillies and Morse, 2005). *In vivo* and *in vitro* studies of hyperpolarized [1-^{13}C] pyruvate or other hyperpolarized tracers (very high sensitivity, restricted time window) (Golman et al., 2006).
Nitrogen (^{15}N)	Low sensitivity due to low gyromagnetic ratio and low endogenous concentration.	*In vivo* and *ex vivo* studies of hyperpolarized ^{15}N-enriched substrates (Cudalbu et al., 2010).
Fluorine (^{19}F)	High sensitivity of detection, but low endogenous concentration. Require use of exogenous tracers.	Assessment of exogenous compounds for studies of enzymatic activity or tumour microenvironment (Le et al., 2009).
Phosphorous (^{31}P)	Medium sensitivity of detection due to medium gyromagnetic ratio and medium concentration in tissue.	Allows studies of phospholipid metabolites, including the phosphomonoesters (PME) phosphoethanolamine (PE) and phosphocholine (PCho) and the phosphodiesters glycerophosphoethanolamine (GPE) and glycerophosphocholine (GPC). The bioenergetics of cancer cells can be studied through ATP/ADP and phosphocreatince levels. *In vivo* studies of tumour hypoxia using the chemical shift of inorganic phosphate (Gillies and Morse, 2005).

sensitivity (Ardenkjaer-Larsen et al., 2003). Rapid heating and dissolution of the polarised sample in a suitable buffer produces an injectable solution. The duration of the polarisation depends on the T_1 of the tracer molecule in the liquid state, but the typical relaxation times of 20–60 s are likely to yield acceptable signal-to-noise ratios also in clinical systems. Following injection of hyperpolarised ^{13}C tracers, the amounts of tracer and its downstream metabolites can be assessed using MRS, providing information on both tracer uptake in the tissue and relative rates of enzymatic conversion of the tracer. This is a fundamentally new approach to imaging of cancer metabolism, which could help understand the relationship between metabolic abnormalities, malignancy and response to therapy. The range of available tracer molecules for this technique is similar to that of positron emission tomography (PET), with [1-^{13}C]-pyruvate as the most studied tracer to date. Despite the increase in sensitivity compared to regular ^{13}C MRS, the sensitivity is inferior to PET, and the amount of tracer needed for imaging is several orders of magnitude larger. This may be an issue both with respect to tracer interference with metabolic pathways and safety of the agents. Current dose levels do not, however, cause significant adverse effects, and a clinical phase I trial of [1-^{13}C] pyruvate in prostate cancer patients has been initiated. For a comprehensive and up to date review of hyperpolarised ^{13}C MRS in cancer metabolism, the authors recommend (Kurhanewicz et al., 2011).

3. Positron emission tomography

For non-invasive monitoring of treatment effects on a molecular level, PET has emerged as an attractive modality. This modality allows dynamic imaging of radionuclide-labelled tracers which interact with metabolic pathways, thereby providing information on the metabolic status of the imaged tissue. Tracer molecules can be labelled with positron-emitting isotopes such as ^{11}C or ^{18}F, which decay and cause annihilation events that can be imaged *in vivo*. Currently, ^{18}F-fluorodeoxyglucose (^{18}F-FDG) is the most frequently used tracer in PET imaging of cancer. FDG is a glucose analogue, and its uptake in cancer cells is representative for the rate of facilitated glucose transport. Inside the cell, it is rapidly phosphorylated by hexokinase but does not undergo further metabolism. ^{18}F-FDG therefore accumulates in cells with high glucose turnover. In preclinical studies, a plethora of tracers have been used to image metabolic features of tumours, including fatty acid metabolism, amino acid uptake and choline metabolism using radiolabelled endogenous substances or analogues of these. PET using metabolic tracers has very high sensitivity, but despite co-acquisition of PET and CT images the relatively poor spatial resolution may be a limitation for the use of PET in therapy monitoring (Brindle, 2008). Another limiting factor in PET is the dependence on tracer kinetics, which is highly variable between organs. The utility of ^{18}F-FDG PET, for example, is restricted in brain cancer (high native glucose uptake) and prostate cancer (low tumour glucose uptake). In addition, infiltrating inflammatory cells may also accumulate ^{18}F-FDG, thereby limiting the sensitivity of PET in assessment of response to therapy (Juweid and Cheson, 2006). Overall, PET and MRS are partly competing, partly complementary modalities as their performance in terms of sensitivity and spatial resolution is fundamentally different. Due to the high specific activity of the radiolabelled tracers used in PET, this method has a detection limit in the picomolar range (Brindle, 2008). The spatial resolution, on the other hand, is ultimately limited by the distance travelled by the positrons prior to annihilation, which is approximately 1 mm. In contrast, *in vivo* ^{1}H MRS is used to measure the levels of metabolites typically present in the millimolar range. The sensitivity is improved in *ex vivo* and *in vitro* MRS, but is limited to the low-micromolar range (Grimes and O'Connell, 2011). The spatial resolution in MRS is often a trade-off between metabolite concentration and the time available for the examination, but spatial resolution down to 1 mm is frequently reported in preclinical volume-localised single-voxel experiments (Fricke et al., 2006; Lei et al., 2010). Hyperpolarised ^{13}C MRSI offers dynamic spectroscopic imaging of tracers present in the micromolar range. Depending on the application, preclinical hyperpolarized ^{13}C MRSI can be performed with a temporal resolution of 2–3 s with a spatial resolution of less than 3 mm (Hu et al., 2010; Larson et al., 2011). A particular advantage of hyperpolarized ^{13}C MRS compared to PET is the possibility of measuring the metabolic fate of the tracer, and that several tracers can be injected simultaneously (Wilson et al., 2010). Using PET, only the total number of decay events is measured, and the counts originating from the tracer cannot be distinguished from those originating from its downstream metabolites. In therapy monitoring, which typically involves serial imaging, the radiation dose due to combined PET/CT examinations may also be a limiting factor.

Overall, both PET and MRS are suitable modalities for non-invasive molecular imaging in cancer. Scientists are in the position to choose from a relatively wide range of endogenous metabolites or exogenous tracers which report on the metabolic activity of a selected tissue. For metabolic imaging, the choice of modality depends on requirements in spatial and temporal resolution, sensitivity and tissue-specific regulation of metabolic pathways, as well as the availability of relevant tracers.

Although this review primarily aims at describing the use of MRS in monitoring response to anticancer therapy, some examples using PET tracers are included in order to illustrate the metabolic effects of some targeted drugs.

4. Oncogenic signalling pathways

The archetypical cancer cell has several characteristics that distinguish it from normal cells, often termed "the hallmarks of cancer" (Hanahan and Weinberg, 2000). These hallmarks are related to the growth, proliferation and survival of cells, but also include the ability to interact with the surrounding tissue. According to this paradigm, changes in a relatively small number of functional properties are sufficient for transformation of a normal cell to a cancer cell. The underlying molecular biology of these properties is, not surprisingly, very complex and clearly beyond the scope of this review. The discovery that proto-oncogenes actually were part of the mammalian genome (Harvey, 1964) has culminated in the characterisation of more than 100 oncogenes and 20 tumour

suppressor genes (Yokota, 2000). It is widely accepted that transformation into a fully malignant cancer cell is a stepwise process involving accumulation of genetic abnormalities (Vogelstein and Kinzler, 2004). Taking the spectrum of genetic abnormalities found in individual cancer patients into consideration, it is not likely that one single targeted drug can effectively inhibit all cancer types. Biomarkers that report on cellular function downstream of oncogenic signalling could increase our understanding of the transduction of signals trough major oncogenic and tumour suppressor pathways and how these pathways interact to cause different subtypes of disease.

Although a high number of individual oncogenes have been described, cancer-specific changes in cellular proliferation and survival are typically assigned to a handful of major oncogenic signalling pathways. Each of these oncogenic signalling pathways include several potential drug targets, and the exact cause of signalling malfunction must be identified before developing targeted drugs. For comprehensive reviews of oncogenic signal transduction pathways and their importance in anticancer therapy, we recommend recent publications by (Bild et al., 2006; Engelman, 2009; Levitzki and Klein, 2010).

4.1. *Oncogenic signalling regulates metabolic pathways*

From a metabolic point of view, the numerous mutations observed in oncogenes cause altered function in a limited number of bioenergetic and biosynthetic pathways. Each oncogenic signalling pathway contains a large number of transduction effectors which, through loss or gain of function, may cause similar downstream effects. These effects include regulation of metabolic enzymes, and each oncogenic signalling pathway can have several different target enzymes within each metabolic pathway. A complete overview of oncogenic regulation of metabolism is therefore beyond the scope of this paper, and the reader is referred to comprehensive reviews for further reading (Dang et al., 2009; Deberardinis et al., 2008; Yeung et al., 2008). A brief overview of oncogenic regulation of phospholipid and glucose metabolism is, however, presented below. Simultaneous dysregulation of these two metabolic pathways is often seen in cancer, due to the fact that important transcription factors such as c-MYC, HIF-1 and p53 regulate a multitude of target proteins involved in cell cycle regulation, production of intermediate metabolites and generation of energy to fuel cellular growth. Interestingly, these transcription factors promote similar changes in metabolism, and abnormal function in these proteins or their upstream regulators will cause cancer cells to converge at a cancer-specific metabolic phenotype. This phenotype includes abnormalities in bioenergetic pathways, such as aerobic glycolysis, *de novo* fatty acid synthesis and glutaminolysis (Deberardinis et al., 2008). In addition, choline metabolism is abnormal in a wide range of cancers (Gillies and Morse, 2005). Key metabolites in both glucose and choline metabolism can be noninvasively studied using MRS, and these metabolites are therefore often suggested as potential pharmacodynamic biomarkers. However, with the emergence of new substrates for hyperpolarised ^{13}C magnetic resonance spectroscopic imaging (MRSI), biomarkers may be found in other pathways as well. Oncogenic regulation of metabolism is an important field of research since endogenous metabolites may serve as biomarkers for response to targeted anticancer drugs. MRS is a versatile tool for non-invasive monitoring of metabolite concentrations in tumours, and the effects of treatment with targeted drugs on phospholipid and glucose metabolism is discussed in the following sections.

5. Choline metabolism

Choline metabolites, such as phosphocholine (PCho) and glycerophosphocholine (GPC), studied by MRS are part of the phosphatidylcholine (PtdCho) pathway. The anabolic and katabolic reactions regulating the amount of this important membrane phospholipid can therefore be studied using MRS. The aetiology of the abnormal choline metabolism in cancer is currently not fully understood. It has, been established that proliferation rate is associated with uptake and phosphorylation of choline in cultured cancer cells, and it has been suggested that this reflects intensified turnover rate of cell membrane components (Gillies et al., 1994; Podo, 1999). It has also been shown that phospholipid metabolites act as second messengers in oncogenic signalling cascades and have inherent mitogenic activity (Cai et al., 1993; Carnero et al., 1994; Cuadrado et al., 1993). Several studies have demonstrated how oncogenic signalling pathways regulate choline metabolism. The uptake of choline into cancer cells is positively regulated by the PI3K/AKT/MTOR pathway (Wang et al., 2007). The tumour microenvironment also influences regulation of choline metabolism. Tumour hypoxia leads to activation of HIF-1, which has been shown to have a direct regulatory effect on the choline metabolism (Glunde et al., 2008). Increased choline uptake is seen in many cancers *in vivo*, allowing the use of ^{11}C or ^{18}F-labelled choline as a tracer in diagnostic positron emission tomography (PET) (Mertens et al., 2010). Using the same tracers, it has been shown that various interventions, such as endocrine therapy or chemotherapy, reduce the uptake of choline, suggesting that PET imaging may be an alternative approach for *in vivo* assessment of metabolic effects of targeted drugs (De et al., 2010; Fei et al., 2010; Krause et al., 2010).

The most typical molecular alteration of choline metabolism seen in cancer is overactivity of choline kinase alpha (CHKA), which phosphorylates choline to PCho (Wu and Vance, 2010). Phospholipases C and D, which hydrolyse PtdCho to PCho and choline, respectively, are also frequently upregulated in cancer (Iorio et al., 2005; Noh et al., 2000). This typically led to abnormally high concentrations of choline, PCho and GPC, but the relationships between regulation of enzymatic activity and metabolite concentrations remain to be understood. Choline kinase overactivity has been associated with overactive *RAS* signalling, mediated through both the PI3K/AKT/MTOR and RAF/MEK/ERK pathways (Ramirez de Molina et al., 2002). The associations between PI3K activity and choline metabolism are shown in Figure 2.

An important link between oncogenic signalling pathways and choline metabolism is the transcription factors MYC and HIF-1. MYC activation induces expression of choline kinase and CTP:phosphocholine cytidylyltrasferase, which have important regulatory roles in synthesis of glycerophospholipids (Morrish et al., 2009, 2008). MYC activation can be caused by

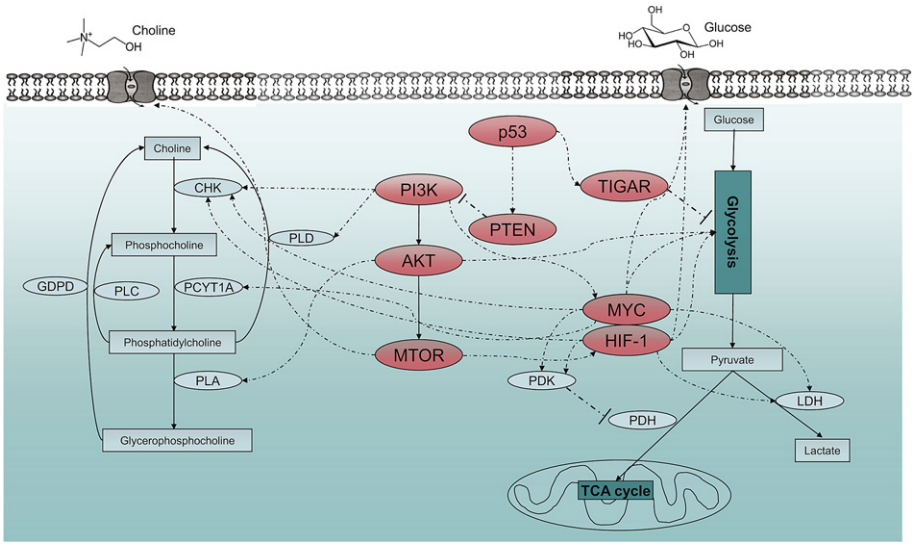

Figure 2 – PI3K signalling regulates both choline and glucose metabolism. The PI3K/AKT/MTOR signalling pathway regulates both choline and glucose metabolism by controlling the activity of key enzymes on different levels in both these metabolic pathways. Elliptic shapes represent proteins whereas rectangular shapes represent metabolites or metabolic pathways. Solid lines represent metabolic conversion, dotted lines represent the regulatory effects from oncogenic signalling transducers (red) on metabolic enzymes (blue). Abbreviations: CHK: choline kinase, PCYT1A: choline phosphate cytidylyl transferase alpha, PLA: phospholipase A, PLC: phospholipase C, PLD: phospholipase D, GDPD: glycerophosphodiester phosphodiesterase, PDK: pyruvate dehydrogenase kinase, PDH: pyruvate dehydrogenase, LDH: lactate dehydrogenase, TCA: tricarboxylic acid, PTEN: phosphatase and tensin homologue; TIGAR: TP53-induced glycolysis and apoptosis regulator, PI3K: phosphatidylinositol-3-kinase, MTOR: mammalian target of rapamycin, AKT: v-akt murine thymoma viral oncogene homologue 1, HIF-1: hypoxia-inducible factor 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

gain-of-function mutations, but accumulation of phosphorylated MYC is also regulated through both the RAS/RAF/MEK/ERK and PI3K/AKT/MTOR cascades (Sears et al., 2000). HIF-1 can be activated both through oncogenic signalling or tumour hypoxia, and has been shown to be a positive regulator of CHKA expression (Glunde et al., 2008). Another central signalling node in cancer is the tumour suppressor p53. This protein acts on metabolism at several different levels, and loss of p53 activity leads to both increased glycolytic rate and increased levels of choline metabolites (Mori et al., 2004; Yeung et al., 2008). In summary, although the mechanisms are not fully understood, several major oncogenic signalling pathways directly or indirectly regulate choline metabolism. On the other hand, inhibition of choline kinase, a key enzyme in phospholipid metabolism has been shown to attenuate RAS/RAF/MEK/ERK and PI3K/AKT/MTOR signalling, demonstrating that there may be a reciprocal relationship between oncogenic signal transduction and choline metabolism, and that PCho or other metabolites involved in choline metabolism may be regarded as oncometabolites (Yalcin et al., 2010).

6. Glucose metabolism

An abnormally high rate of glucose uptake and utilisation is seen in most cancers. In contrast to normal cells, cancer cells extract energy from glucose through glycolysis rather than oxidative phosphorylation, even under normoxic conditions (Warburg, 1924). The low ATP yield is compensated by a high metabolic flux (Vander Heiden et al., 2009). This way, cancer cells can produce energy while conserving carbon for production of proteins and nucleotides, as required to maintain a high rate of proliferation. The oncogenic signalling pathways that regulate choline metabolism have also been shown to regulate glucose metabolism (Elstrom et al., 2004; Lock et al., 2011; Tennant et al., 2009; Yeung et al., 2008). PI3K-mediated effects are presented in Figure 2, including the effects mediated by MYC and HIF-1. Glucose metabolism is also controlled on several levels by p53 as well as by *VSRC* and *RAS*-mediated effects, (Dang and Semenza, 1999; Levine and Puzio-Kuter, 2010). This demonstrates how overactivity in a variety of oncogenic signalling pathways converge at a common phenotype, which apparently is beneficial for cancer cell proliferation and survival. The cancer-specific aerobic glycolytic metabolism is associated with upregulation of several key enzymes in glycolysis. Firstly, increased transmembrane glucose transport is facilitated by increased levels of glucose transporters, primarily GLUT1. Glycolytic genes, including hexokinase and pyruvate kinase are also upregulated (Altenberg and Greulich, 2004). Finally, upregulation of lactate dehydrogenase (LDH) allows production of large amounts of lactate. Intermediate glycolytic metabolites are generally present in low concentrations and cannot be detected using MRS. In contrast, lactate concentration in malignant disease is generally high, allowing the use of lactate as a MRS biomarker. Intermediate metabolites of glycolysis and the Krebs cycle can in some cases be studied using MRS after administration of ^{13}C-enriched

substrates (Ikehira et al., 1990; Williams and Gadian, 1986). After a lag time, concentrations of downstream metabolites can be measured using ^{13}C MRS. This has been used for *in vivo* studies of response to endocrine therapy in breast cancer xenografts, but the method is limited by the need for large quantities of ^{13}C-enriched glucose (Rivenzon-Segal et al., 2003). A more sensitive approach is to measure relative rates of enzymatic activity using hyperpolarized ^{13}C MRSI. Although restricted by the relaxation rate of hyperpolarized substrates, this method allows real-time metabolic imaging of substrates such as [1-^{13}C] pyruvate, [2-^{13}C] fructose, [5-^{13}C] glutamine and $H^{13}CO_3^-$ (Gallagher et al., 2008; Golman et al., 2006; Keshari et al., 2009). Hyperpolarized choline is also a potential substrate for metabolic imaging, potentially allowing *in vivo* assessment of choline kinase activity in cancer (Allouche-Arnon et al., 2010; Cudalbu et al., 2010; Gabellieri et al., 2008).

The effect of targeted drugs on glucose metabolism has also been investigated using ^{18}F-fluorodeoxyglucose (FDG) PET, emphasising that reduced glucose transport and hexokinase activity is associated with response to cytostatic treatments and that PET and MRS may play complementary roles in assessing therapy of targeted drugs (Contractor and Aboagye, 2009). Studies of glucose and choline metabolism using PET are briefly discussed in this review, as it is likely that changes observed using FDG or choline derivatives as tracers in PET also can be observed using MRS. Choline metabolism in cancer has also been studied *in vitro* and *in vivo* using ^{13}C MRS following administration of [1,2-^{13}C] choline, demonstrating the feasibility of this approach for assessment of response to therapy (Katz-Brull et al., 2001).

7. Receptor protein kinases

Several oncogenic signalling cascades are overactive due to abnormal function of receptor protein kinases. Blocking these receptors, either by ligand scavenging, receptor antagonism or direct inhibition of the catalytic domain, may reduce downstream signalling or inhibit tumour growth.

7.1. BCR-ABL

One of the best understood genetic abnormalities in cancer is the chromosomal translocation between chromosome 9 and 22 forming the Philadelphia chromosome. This result of this defect is production of the fusion oncoprotein BCR-ABL, which is the single abnormality driving early CML. BCR-ABL contains a receptor tyrosine kinase domain which is constitutively activated, and a plethora of downstream targets have been identified. These include both the PI3K/AKT/MTOR and RAS/RAF/MEK/ERK pathways, causing both evasion of apoptosis and increased proliferation (Kharas et al., 2004; McCubrey et al., 2007; Puil et al., 1994). As BCR-ABL only is present in malignant cells, it is an attractive drug target. The kinase inhibitor imatinib (Gleevec®, Novartis) was approved for treatment of CML in 2001, probably being the first receptor tyrosine kinase inhibitor to enter the clinic. Imatinib blocks BCR-ABL activity, but also inhibits CKIT and platelet-derived growth factor receptor (PDGFR). The effect of imatinib on lymphocyte metabolism has been studied using several different methods. Using [1-^{13}C] glucose MRS, it has been shown that imatinib inhibits glucose uptake in BCR-ABL-positive cells, but not in BCR-ABL negative cells (Gottschalk et al., 2004). In addition, glucose metabolism was shifted from production of [3-^{13}C] lactate in favour of [4-^{13}C] glutamate, indicating specific inhibition of anaerobic glycolysis associated with increased utilisation of glucose for oxidative phosphorylation. Choline metabolism has been studied using ^{31}P MRS, demonstrating a decreased PCho signal and increased GPC and GPE signals (Klawitter et al., 2009). In imatinib-resistant CML cell lines, however, imatinib did not induce the same changes in metabolism. The effect of imatinib on glucose metabolism has been further investigated *in vivo* using hyperpolarized [1-^{13}C] pyruvate MRSI (Dafni et al., 2010). In PC3/MM2 prostate canecer xenografts, imatinib inhibits PDGFR activity, resulting in reduced LDH activity and reduced conversion of pyruvate to lactate. The metabolic changes were accompanied by reduced HIF-1 and MYC activity, demonstrating the direct links between oncogenic signalling and energy metabolism. Representative MRS spectra from *in vitro* and *in vivo* experiments with imatinib are presented in Figure 3. Imatinib has also been used in treatment of gastrointestinal stromal tumours (GISTs). This cancer frequently harbours gain-of-function mutation in *CKIT* or *PDGFR*, which both are inhibited by imatinib. Several studies have shown that reduced ^{18}FDG uptake is associated with response to imatinib therapy, and that lack of change in ^{18}FDG uptake is associated with disease progression and poor survival (Contractor and Aboagye, 2009; Holdsworth et al., 2007).

In patients not responding to imatinib, a decrease in ^{18}FDG uptake has been observed after treatment with the vascular endothelial growth factor receptor (VEGFR)/PDGFR/CKIT inhibitor sunitinib (Prior et al., 2009). In melanoma patients with activating *CKIT* mutation, dasatinib treatment has also caused a response to treatment accompanied by decrease in ^{18}FDG uptake (Woodman et al., 2009).

7.2. HER-2

The human epidermal growth factor receptor (HER-2) is overexpressed in a subtype of breast cancer due to amplification of the *HER-2* gene. Downstream targets include the PI3K/AKT and RAF/MEK/ERK pathways (Sergina and Moasser, 2007). The anti-HER-2 antibody trastuzumab targets the extracellular domain of the receptor and blocks its activity. This drug has been shown to prolong the life of patients with HER-2-positive breast cancer, but inherent or acquired resistance to therapy is a clinical challenge (Mukai, 2010). Using ^{18}FDG PET, it has been shown that reduced glucose uptake is associated with response to trastuzumab therapy in some, but not all, animal models of HER-2-expressing breast cancer (McLarty et al., 2009; Shah et al., 2009). Using [^{11}C] choline as tracer, response to treatment was associated with reduced uptake of choline, suggesting a potential for non-invasive therapy monitoring (Kenny et al., 2010). The effect of trastuzumab on choline metabolism has also been investigated in transgenic mice using ^{31}P MRS, demonstrating an increase in PME/nucleotide triphosphate (NTP) ratio in treated tumours. As NTP levels increased relative to the total accumulated ^{31}P signal, increased PCho

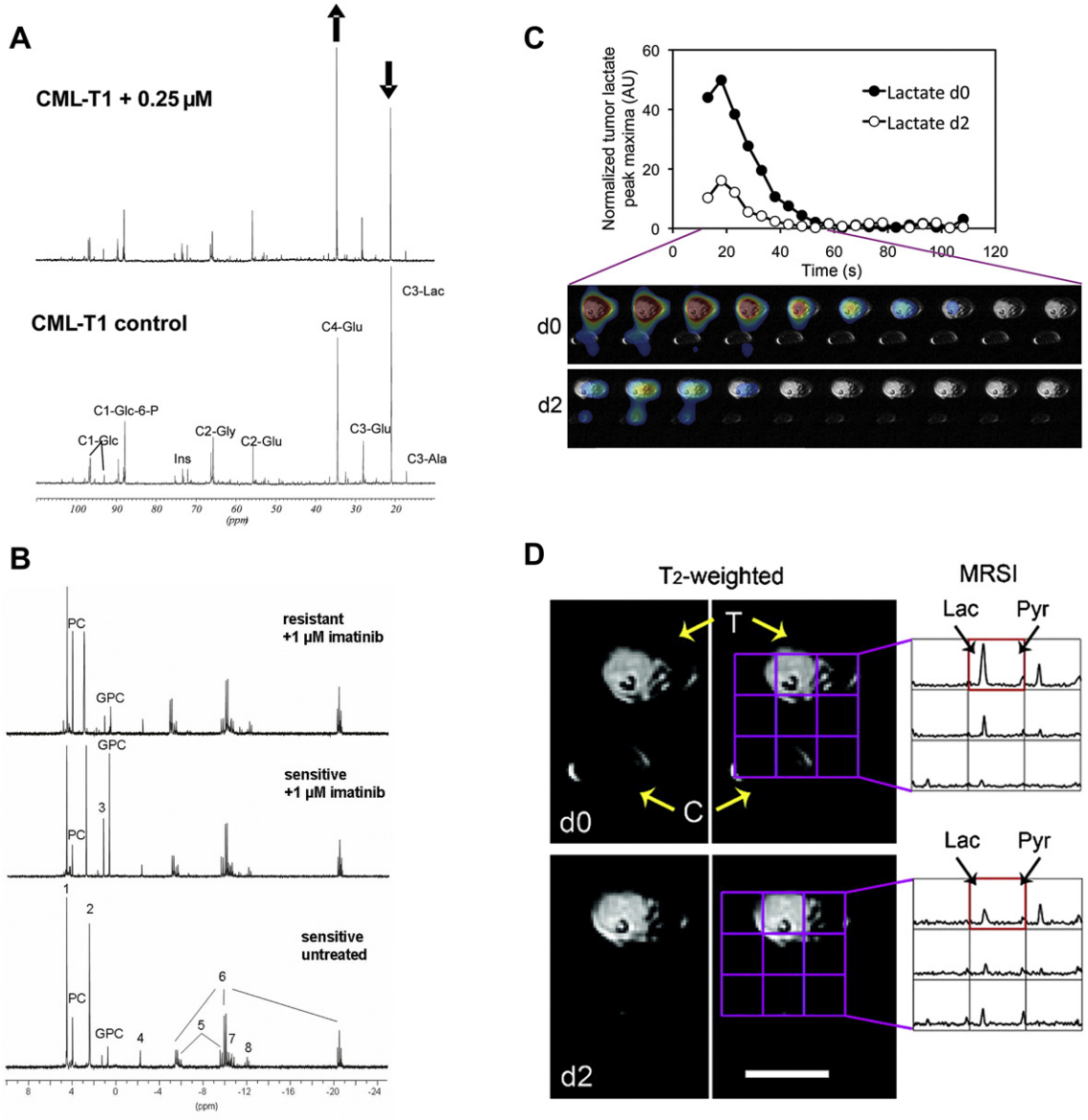

Figure 3 – The effect of imatinib on choline and glucose metabolism. A: ^{13}C MR spectra of *BCR-ABL* positive CML cells 4 h after addition of 5 mM [1-^{13}C] glucose. Top: Extract obtained 96 h after incubation with 0.25 μM imatinib. Bottom: Control extract. Imatinib-treated cells produce less [3-^{13}C] lactate and more [4-^{13}C] glutamate than controls. B: ^{31}P MR spectra of *BCR-ABL* positive CML cells resistant (top) and sensitive (middle) to imatinib treatment. Resistant cells had a similar choline metabolite profile as untreated controls (bottom). Sensitive cells had decreased PCho and increased GPC concentration 24 h after incubation with 1 μM imatinib. C: Time-course of hyperpolarized [1-^{13}C] lactate production before (full circles) and 2 days after (open circles) treatment with 50 mg/kg/day imatinib, obtained after injection of hyperpolarized [1-^{13}C] pyruvate in a xenograft model of prostate cancer. D: *In vivo* MRSI spectral arrays from the same experiment, demonstrating more extensive lactate production in tumour regions compared to surrounding tissue, and a reduction in peak lactate concentration in the xenograft 2 days after imatinb treatment. Illustrations adapted and reprinted with permission from the American Association for Cancer Research and John Wiley & Sons Ltd. (Dafni et al., 2010; Gottschalk et al., 2004; Klawitter et al., 2009).

may be the most plausible explanation for this change (Rodrigues et al., 2004).

7.3. PI3K/AKT/MTOR

Overactivity of the PI3K/AKT/MTOR pathway can be caused either by loss of tumour suppressor gene function (p53 or PTEN) or gain of PI3K function, and causes both increased glucose uptake, increased glycolytic flux and a switch from mitochondrial respiration to lactate production (Barthel et al., 1999; Elstrom et al., 2004; Matoba et al., 2006). Interestingly, blockade of PI3K/AKT/MTOR also reduce choline kinase and fatty acid synthase (FAS) activity, demonstrating the global metabolic effects of intervening with oncogenic signalling (Al-Saffar et al., 2010). Genetic alterations in this pathway are observed in a wide range of cancers. The metabolic effects

of several different PI3K inhibitors have been studied by MRS, predominantly *in vitro*. Early model substances such as wortmannin and LY294002 were studied by Beloueche-Babari et al in cultured breast cancer cells with mutations in *NRAS* (MCF-7), *KRAS* (MDA-MB-231) and *HRAS* (Hs578T) (Beloueche-Babari et al., 2006). The MDA-MB-231 and Hs578T cell lines also had mutated *p53*. Using ^{31}P MRS, it was found that LY294002, a reversible inhibitor of PI3K, reduced PCho in all tested cell lines. In MDA-MB-231 cells, an increase in GPC was also found. In contrast, wortmannin, an irreversible inhibitor of PI3K (Arcaro and Wymann, 1993), reduced PCho without increasing GPC. This difference was attributed to differences in target specificity. *In vivo*, the PI3K inhibitor PX-866 has been shown to reduce the tCho signal in a xenograft model of glioblastoma, demonstrating the potential for translation of therapy monitoring using MRS to the clinic (Koul et al., 2010). The effect of PI3K inhibition using LY294002 has also been studied by hyperpolarized [1-^{13}C] pyruvate MRSI (Ward et al., 2010). In cultured MDA-MB-231 and GS-2 (glioblastoma) cells, a decrease in LDH activity was accompanied by reduction in formation of [1-^{13}C] lactate. In the same experiments, ^{31}P MRS confirmed a reduction of both PCho and PE concentration following PI3K inhibition. The metabolic changes were associated with reduced activity both in the PI3K signalling pathway and its downstream targets HIF-1 and LDH. The reduction in lactate formation rate was also seen in GS-2 cells after treatment with the MTOR inhibitor everolimus, and these findings translated into a drop in lactate:pyruvate ratio *in vivo*. This is a significant result, suggesting that *in vivo* downregulation of glucose metabolism observed using FDG-PET is likely to include reduction in LDH activity, and that this can be monitored using hyperpolarized ^{13}C MRS (Engelman et al., 2008; Pantaleo et al., 2010; Wei et al., 2008).

7.4. RAS/RAF/MEK/ERK

Another major oncogenic signalling pathway that has been positively associated with modulation of choline metabolism is the RAS/RAF/MEK/ERK system (Bjorkoy et al., 1997; Lacal, 1990; Lacal et al., 1987). In particular, increased activity of choline kinase is mediated by RAS. This activation is modulated both trough the PI3K pathway and the RAL-GDS effector (Ramirez de Molina et al., 2002, 2001). Choline kinase has also been proposed as an upstream regulator of RAS/RAF/MEK/ERK signalling, as shown by Yalcin et al. (2010). This may at least in part explain the tumour-inhibiting properties of choline kinase inhibitors such as hemicholinium-3 and MN58b (Ramirez de Molina et al., 2004).

Using MRS, it has been shown that transfection of NIH3T3 cells with mutated *HRAS* caused increased levels of PCho, an effect reversed by treatment with simvastatin and 17-AAG (Ronen et al., 2001). These drugs target RAS signalling through different mechanisms. Simvastatin, a HMG-CoA transferase inhibitor, is thought to cause depletion of a farnesyl moiety needed for anchoring RAS to the cell membrane. 17-AAG is a heat shock protein 90 (HSP90) binding protein which cause depletion of several proteins including RAF, a signal transducing protein downstream of RAS. As key components in the RAS/RAF/MEK/ERK pathway frequently are mutated and constitutively active in many cancers, inhibition of this signal transduction is, in principle, a promising strategy for development of anticancer drugs. However, no drugs acting through direct inhibition of RAS activity have yet been approved for clinical use. Due to the complexity of this pathway both with respect to inherent regulation of signal transduction as well as cross-talk with other pathways, the number of drugs acting through inhibition of downstream transducer proteins remains limited. Specific inhibition of the MEK1 kinase with U0126 in cultured breast and colon carcinoma cells also caused a reduction in PCho concentration, demonstrating that this metabolite is a potential biomarker for drugs targeting the RAS/RAF/MEK/ERK axis (Beloueche-Babari et al., 2005). Using PET, it was shown that inhibition of cyclin D kinase (CDK) with PHA-848125 in transgenic mural lung cancer with gain-of-function *KRAS* mutation reduced tumour growth and uptake of [^{11}C] choline (Degrassi et al., 2010).

Inhibition of choline kinase has demonstrated preclinical efficacy in several cancers. In studies where siRNA against the enzyme has completely abolished PCho production, RAS/RAF/MEK/ERK and PI3K signalling has also been attenuated (Yalcin et al., 2010). In contrast, incomplete inhibition of choline kinase using the small molecule inhibitor MN58b has inhibited tumour growth without concomitant effects on these signalling pathways (Rodriguez-Gonzalez et al., 2003). In both cases, significant reduction of PCho was observed, a finding that has been reproduced using *in vivo* ^{31}P MRS in both breast and colon cancer xenografts (Al-Saffar et al., 2006; Glunde et al., 2005).

As mentioned above, drugs targeting HSP90 cause cytostasis via several onocogenic signalling pathways, including the RAS/RAF/MEK/ERK system. HSP90 is a chaperone associated with multiple post-translational activities, and is essential for the stability and function of many oncogenic signalling proteins (Powers and Workman, 2006). Inhibitors of HSP90 therefore modulate cancer cell metabolism through several different molecular targets, including AKT, BCR-ABL, RAF and HIF-1 (Maloney and Workman, 2002). It is therefore not entirely surprising that various metabolic effects of HSP90-inhibitors have been observed in different experimental systems. In NIH3T3 cells transfected with mutant *HRAS*, the HSP90-inhibitor 17-AAG caused a decrease in PCho concentration (Ronen et al., 2001). In contrast, the same inhibitor caused increased PCho and GPC in cultured colon cancer cells and increased levels of phosphomonoesters (PCho + PE) in HT29 colon cancer xenografts (Chung et al., 2003). *In vivo* studies of CWR22 prostate cancer xenografts demonstrated a reduction in tCho following 17-AAG (Le et al., 2009). A recent study in human melanoma cells with wild-type *BRAF* (CHL-1) and mutated *BRAF* (SKMEL28) showed that 17-AAG and CCT018159 showed reduced RAF/MEK/ERK signalling following HSP90 inhibition, associated with a 2.5-fold increase in GPC concentration but no change in PCho (Beloueche-Babari et al., 2010). GPC accumulation was prevented by inhibition of phospholipase A2, suggesting that inhibition of HSP90 modulates choline metabolism also through this enzyme. This is supported by findings in breast cancer cells, where both choline, PCho and GPC concentrations increased following 17-AAG treatment. The increase in choline and PCho was associated with increased expression of the choline transporter SLC44A1, but altered

activity of phospholipases A2, C and D also was also observed (Brandes et al., 2010). This variety of metabolic effects exerted by HSP90 inhibition probably reflects differences between cancers in the relative importance of different oncogenic drivers, which in turn cause differences in downstream cellular and metabolic effects. The present studies of HSP90 inhibition nicely exemplify the complexity of metabolic changes caused by targeted therapy, demonstrating how metabolic response is dependent on context.

Despite completely different mechanisms of action, histone deacetylase (HDAC) inhibitors have a similar effect on oncogenic signalling as HSP90-inhibitors. HDAC inhibitors alter transcriptional activity of nuclear transcription factors with relevance in cancer, including CMYC, nuclear factor κB (NFκB), HIF-1α and oestrogen receptor α (Lane and Chabner, 2009). Interestingly, HDAC inhibitors also block the action of HSP90 by inducing hyperacetylation of this protein, thereby increasing the proteosomal degradation of oncogenic proteins such as BCR-ABL, epidermal growth factor receptor (EGFR), HER-2, AKT and CRAF (Lane and Chabner, 2009). Studies of metabolic changes in HT29 colon cancer cells using the HDAC inhibitors LAQ824 and SAHA have shown increased PCho concentrations without concomitant increase in GPC (Chung et al., 2008). In tumour extracts from the same experiments, it was found that both PCho and PE levels were increased, whereas GPC and glycerophosphoethanolamine (GPE) were decreased following LAQ824 treatment (Chung et al., 2008). Increased PCho has also been observed in prostate cancer cells treated with a fluorinated SAHA analogue (Sankaranarayanapillai et al., 2006). The less specific HDAC inhibitor phenylbutyrate, on the other hand, increased GPC but not PCho (Milkevitch et al., 2005). The increase in GPC was enhanced by the HMG-CoA reductase inhibitor lovastatin, suggesting that additional modulation of RAS signalling by this compound contributed to the metabolic response (Milkevitch et al., 2007).

8. Transcription factors

As oncogenic signalling pathways ultimately regulate cellular processes through altered transcription of genes, the transcription factors (TFs) facilitating this altered expression of genes are prospective drug targets in cancer. The number of drugs targeting TFs is, however, still limited. Tamoxifen, an inhibitor of the oestrogen receptor (ER) was developed without knowledge about its molecular target, but still serves as an example of the therapeutic potential in small molecules inhibiting TFs. Activation of the ER receptor by oestrogen leads to transcription of a number of genes involved in cell proliferation, and although *de novo* or acquired resistance is a problem, tamoxifen has played a significant role in treatment and prevention of breast cancer over the last 30 years (Osborne and Schiff, 2005). Metabolic response to ER blockade has been studied using both *in vitro* and *in vivo* MRS, with [1-^{13}C] glucose as tracer. These studies have shown a decrease in glucose uptake, glycolytic rate and lactate production after tamoxifen treatment (Furman et al., 1992; Rivenzon-Segal et al., 2003). The reduced glucose utilisation was associated with a reduction in glucose GLUT1 expression. In these experiments, no treatment-related changes in choline metabolism were observed (Furman et al., 1992).

Another transcription factor that can be targeted in cancer treatment is HIF-1. Under hypoxic conditions, expression of HIF-1 promotes survival of cancer cells through stimulation of glycolysis as well as neoangiogenesis. There is evidence that HIF-1 is directly regulated by oncogenic signalling cascades as it is upregulated also in leukaemias, where the cells generally do not experience hypoxia (Elstrom et al., 2004). Inhibition of the HIF-1α subunit has been shown to inhibit growth across a wide range of experimental cancer models and cause radio sensitisation in human pancreatic cancer (Schwartz et al., 2010; Welsh et al., 2004). Interestingly, HIF-1 inhibition does not only reduce glucose consumption and glycolytic activity, but also modulates choline metabolism. Analyses of colon cancer xenograft extracts by ^{31}P MRS, showed that PX-478 reduced the concentrations of PCho, GPC, PE and GPE. This finding corresponded with a reduced tCho signal *in vivo* (Jordan et al., 2005). These experiments illustrate how glucose and choline metabolism are regulated through the same mechanisms, and that potential biomarkers may be found in either of these metabolic pathways when upstream signal transducers are targeted in cancer therapy (Jordan et al., 2005).

9. Metabolic pathways

As malignant transformation very often is accompanied by a cancer-specific metabolic transformation, the metabolic pathways themselves contain a plethora of potential anticancer drug targets. In particular, there has been great interest in drugs specifically inhibiting glycolytic enzymes or fatty acid synthesis, and several candidates are now in clinical trials (Tennant et al., 2010). Not surprisingly, the effects of such drugs may be studied by MRS. In the case of glycolysis, drugs targeting hexokinase and thereby reducing glycolytic flux have not been particularly successful as monotherapeutic agents, but may increase the efficacy of other anticancer agents (Maschek et al., 2004; Rosbe et al., 1989; Singh et al., 2005). Several studies have demonstrated that MRS can be used to monitor the metabolic changes induced by this class of drugs. For example, 2-deoxyglucose reduces glycolytic rate *in vitro* through competition with glucose for the hexokinase enzyme (Ben-Horin et al., 1995). Lonidamine, on the other hand, exerts its antineoplastic effect through inhibition of lactate efflux and concomitant intracellular acidification, a finding that has been studied using both ^{31}P MRS (acidification), ^{13}C MRS and diffusion-weighted ^{1}H MRS (lactate build-up) (Ben-Horin et al., 1995; Mardor et al., 2000). Glucose metabolism is also modulated by dichloroacetate, which has shown remarkable anticancer effects both in preclinical models and in clinical trials (Michelakis et al., 2010; Sun et al., 2010). This drug redirects pyruvate via acetyl-CoA into the tricarboxylic acid (TCA) cycle by activating the pyruvate dehydrogenase (PDH) complex (Vander Heiden, 2010). Although the exact mechanism for cell death is uncertain, PDH activation apparently renders cancer cells unable to meet their bioenergetic and biosynthetic needs. The shift in the metabolic fate of glucose has been studied using [1-^{13}C] glucose MRS, demonstrating decreased lactate

production and increased levels of ^{13}C-labelled TCA intermediates (Simpson et al., 2006).

The effect of metabolic inhibitors on choline metabolism has also been demonstrated using MRS. FK866, an inhibitor of nicotinamide phosphoribosyltransferase, induce apoptosis through depletion of NAD^+ and has shown efficacy across a wide range of preclinical models (Hasmann and Schemainda, 2003). In addition to changes in glucose metabolism, a decrease in PCho and an increase in GPC have been associated with response to FK866 treatment (Muruganandham et al., 2005). Inhibition of fatty acid synthase (FAS), which often is upregulated in cancer, with orlistat also cause a MRS-detectable drop in PCho concentration. Using [1-^{13}C] glucose and [1,2-^{13}C] choline as metabolic tracers *in vitro*, it has been shown that orlistat decrease choline kinase activity and reduce *de novo* PCho production (Ross et al., 2008).

Finally, the choline metabolism itself represents a potential target for anticancer therapy (Lacal, 2001). Inhibition of choline kinase has been shown to inhibit tumour growth in preclinical models, and this effect is accompanied by a drop in PCho concentration (Al-Saffar et al., 2006; Clem et al., 2011). It has been suggested that high levels of PCho are associated with downstream production of phosphatidic acid, which in turn acts as a positive regulator of *RAS* signalling. The choline kinase antagonist CK37 has been shown to suppress both RAF/MEK/ERK and PI3K/AKT signalling, demonstrating the complex relationship between the choline metabolism and oncogenic signalling pathways (Clem et al., 2011).

10. The role of metabolic biomarkers in drug development and clinical disease management

The studies described above (summarised in Table 3) demonstrate that drugs inhibiting oncogenic signal transduction cause changes in both glucose and choline metabolism, and that these changes can be monitored *in vitro* and *in vivo* using MRS. The changes in choline metabolism tend to be more complex and are strongly dependent on the type of treatment. The most widely investigated choline metabolism biomarker is PCho, and most studies suggest that a decrease in PCho is correlated with response to treatment. However, in the case of HDAC and HSP90 inhibition, reduction in tumour growth rate is associated with increased PCho concentration. Changes in GPC concentration tend to be even more variable and context-dependent. Furthermore, it has been shown that different subtypes of cancer can have distinct choline metabolite profile due to differences in expression of genes involved in choline metabolism (Moestue et al., 2010). These differences can be caused by microenvironmental factors or the molecular/mutational profile of the cancer subtypes. Nevertheless such differences are likely to have impact on how choline metabolism is altered in response to targeted therapy. This is exemplified in response to HSP90 treatment, where PCho concentration has been found to increase in colon and breast cancer cell lines, but decrease in cultured *RAS*-transfected fibroblasts and prostate cancer xenografts. Patients with differences in their molecular profile are likely to benefit from different drugs (or combinations of drugs) and the introduction of targeted anticancer drugs therefore represents a major step into the paradigm of personalised medicine. In order to use choline metabolites as biomarkers, there is therefore a need to understand the relationship between oncogenic signalling and metabolic profile in individual patients. If successful, this may lead to the development of "personalised biomarkers" in the choline metabolism, based on the molecular profile of the patient and the expected response to an individualised treatment regimen. Implementation of personalised biomarkers in clinical medicine represents a big challenge, and it is likely that choline metabolites may play a more important role in preclinical drug development than in clinical therapy monitoring. In preclinical studies, the response in standardised, molecularly well-defined models can be quantified and related to long-term outcome, which is valuable information in the process of lead candidate selection and evaluation. Improved *in vivo* ^{31}P MRS may be an important step along the way, as this modality provides *in vivo* assessment of individual choline metabolites, in contrast to ^{1}H MRS which only allows tCho measurements. However, when considering the use of choline-containing compounds as biomarkers for therapeutic efficacy *in vivo*, the lack of specificity should be taken into account. Reduced PCho concentrations are associated with cell cycle status, induced cell death and reduced fraction of viable cancer cells, as well as changes in oncogenic signal transduction (Anthony et al., 1999). Translation of *in vitro* observations to *in vivo* systems is therefore difficult. Distinguishing between primary and secondary treatment effects may be a problem, especially in cases where these have opposite effects on a biomarker (Brindle, 2008).

From a biological perspective, it is a striking difference between choline metabolism and glucose metabolism. Glucose uptake, glycolytic rate and lactate production is invariably reduced as a response to treatment with targeted anticancer drugs. Therefore, universal biomarkers which can be utilised across cancer subtypes and treatments are more likely to be found in this pathway. Biomarkers related to glucose metabolism may be relatively non-specific and dependant on both vascular function, the fraction of viable cells in a tumour and oncogenic regulation of glycolytic activity. Still, rational use of these biomarkers in evaluating cellular and tumoural response to treatment is possible, provided the biology of the model system is well understood. However, MRS assessment of glucose metabolism *in vivo* is currently a challenge due to low sensitivity and long acquisition times. The emergence of hyperpolarized ^{13}C MRSI will allow rapid and more detailed analysis of metabolic rates, and this method has the potential to be of great value both in preclinical efficacy studies and, in a longer perspective, in clinical therapy monitoring. Hyperpolarised MRSI has some methodological similarities with PET imaging, especially in the high sensitivity and a potential wide range of available metabolic tracers. In addition, the method is more suitable than PET for the use in therapy monitoring, as ionising radiation is not involved. Clinical phase I studies of hyperpolarized [1-^{13}C] pyruvate have recently been initiated, suggesting that translation of this advanced modality into clinical practice is technically feasible.

As shown in this review, the metabolic response to treatment depends both on the mechanism of oncogenic pathway

Table 3 – Effects of molecularly targeted drugs on metabolite concentrations and pathway regulation. MCTs = monocarboxylate transporters, PDK = pyruvate dehydrogenase kinase, NPT = nicotinamide phosphoribosyltransferase, Cho = choline, PCho = phosphocholine, GPC = glycerophosphocholine, PE = phosphoethanolamine, PME = phosphomonoesters, PtdCho = phosphatidylcholine, tCho = choline + phosphocholine + glycerophosphocholine, G6P = glucose-6-phosphate, G3P = glucose-3-phosphate.

Pathway	Drug	Target	Choline metabolism	Glucose metabolism	In vivo/ *in vitro*	References
PI3K/Akt/mTOR	LY294002	PI3K	PCho↓ PE↓ GPC↑	Lactate↓	*In vitro*	(Beloueche-Babari et al., 2006; Ward et al., 2010)
	Wortmannin	PI3K	PCho↓		*In vitro*	(Beloueche-Babari et al., 2006)
	PX-866	PI3K	tCho↓[a]		*In vivo*	(Koul et al., 2010)
	Everolimus	MTOR		Glucose↓[b] Lactate↓	*In vitro/in vivo*	(Pantaleo et al., 2010; Ward et al., 2010)
	PI103	PI3K + MTOR	PCho↓ tCho↓		*In vitro*	(Al-Saffar et al., 2010)
	BEZ235	PI3K + MTOR		Glucose↓[a]	*In vivo*	(Engelman et al., 2008)
Ras/Raf/MekErk	PHA-848125	CDK	Cho↓[c]		*In vivo*	(Degrassi et al., 2010)
	Simvastatin	HMG-CoA	PCho↓		*In vitro*	(Ronen et al., 2001)
	U0126	MEK1	PCho↓		*In vitro*	(Beloueche-Babari et al., 2005)
	MN58b	ChK	PCho↓ tCho↓		*In vitro/in vivo*	(Al-Saffar et al., 2006; Glunde et al., 2005)
	17-AAG	HSP90	tCho↓ Cho↑ PCho↓ ↑ GPC↑ PME↑		*In vitro/in vivo*	(Beloueche-Babari et al., 2010; Brandes et al., 2010; Chung et al., 2003; Le et al., 2009; Ronen et al., 2001)
	CCT018159	HSP90	GPC↑		*In vitro*	(Beloueche-Babari et al., 2010)
	SAHA	HDAC	PCho↑		*In vitro*	(Chung et al., 2008; Sankaranarayanapillai et al., 2006)
	LAQ824	HDAC	PCho↑ Cho ↑ GPC↓ PME↑	Glucose↓	*In vitro/in vivo*	(Chung et al., 2008)
	Phenylbutyrate	HDAC	GPC↑ tCho↑		*In vitro*	(Milkevitch et al., 2005)
Receptor protein kinases	Imatinib	BCR-Abl, c-KIT, PDGFR	PCho↓ PtdCho↓ GPC↑ GPE↑	Lactate↓ Glucose↓	*In vitro/in vivo*	(Contractor and Aboagye, 2009; Dafni et al., 2010; Gottschalk et al., 2004; Klawitter et al., 2009)
	Trastuzumab	HER-2	Cho ↓[c] PME↑		*In vivo*	(Kenny et al., 2010; Rodrigues et al., 2004)
	Dasatinib	BCR-Abl, c-SRC		Glucose↓[b]	*In vivo*	(Woodman et al., 2009)
	Sunitinib	VEGFR, PDGFR, c-KIT		Glucose↓[b]	*In vivo*	(Prior et al., 2009)
Transcription factors	PX-478	HIF-1α	tCho↓ PCho↓ GPC ↓ PE↓ GPE↓		*In vivo*	(Jordan et al., 2005)
	Tamoxifen	ER	No changes	Glucose↓ Lactate↓	*In vitro/in vivo*	(Furman et al., 1992; Rivenzon-Segal et al., 2003)
Metabolic inhibitors	2-deoxyglucose	Hexokinase		Glucose↓ Lactate↓	*In vitro*	(Ben-Horin et al., 1995)
	Lonidamine	MCTs		Lactate↑	*In vitro*	(Ben-Horin et al., 1995; Mardor et al., 2000)
	Dichloroacetate	PDK		TCA intermediates↑	*In vitro*	(Simpson et al., 2006)
	FK866	NPT	PCho↓ GPC↑	G6P↑ G3P↑	*In vivo*	(Muruganandham et al., 2005)
	Orlistat	FAS	PCho↓		*In vitro*	(Ross et al., 2008)
	Mn58b	CHK	PCho↓		*In vitro/in vivo*	(Al-Saffar et al., 2006)
	CK37	CHKA	PCho↓		*In vitro*	(Clem et al., 2011)

a Measured by *in vivo* ^{1}H MRS.
b Measured using ^{18}FDG positron emission tomography.
c Measured using ^{11}C-choline positron emission tomography.

activation and target of the chosen drug. Biomarker behaviour therefore varies between cancers and treatments, and the metabolic response to treatment may be difficult to predict. Biomarker validation in clinically relevant disease models is therefore crucial for clinical implementation. The introduction of hyperpolarised MRSI may allow non-invasive monitoring of glucose metabolism at clinical MR scanners in the near future. Through rational use of different MRS methods, the association between oncogenic signalling and metabolic abnormalities will gradually be better understood, hopefully accelerating the introduction of targeted anticancer drugs into the clinic.

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