Journal of Enzyme Inhibition and Medicinal Chemistry

http://informahealthcare.com/enz
ISSN: 1475-6366 (print), 1475-6374 (electronic)

J Enzyme Inhib Med Chem, Early Online: 1–33
© 2014 Informa UK Ltd. DOI: 10.3109/14756366.2014.966704

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REVIEW ARTICLE

Targeting tumour hypoxia to prevent cancer metastasis. From biology, biosensing and technology to drug development: the METOXIA consortium

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Abstract

The hypoxic areas of solid cancers represent a negative prognostic factor irrespective of which treatment modality is chosen for the patient. Still, after almost 80 years of focus on the problems created by hypoxia in solid tumours, we still largely lack methods to deal efficiently with these treatment-resistant cells. The consequences of this lack may be serious for many patients: Not only is there a negative correlation between the hypoxic fraction in tumours and the outcome of radiotherapy as well as many types of chemotherapy, a correlation has been shown between the hypoxic fraction in tumours and cancer metastasis. Thus, on a fundamental basis the great variety of problems related to hypoxia in cancer treatment has to do with the broad range of functions oxygen (and lack of oxygen) have in cells and tissues. Therefore, activation–deactivation of oxygen-regulated cascades related to metabolism or external signalling are important areas for the identification of mechanisms as potential targets for hypoxia-specific treatment. Also the chemistry related to reactive oxygen radicals (ROS) and the biological handling of ROS are part of the problem complex. The problem is further complicated by the great variety in oxygen concentrations found in tissues. For tumour hypoxia to be used as a marker for individualisation of treatment there is a need for non-invasive methods to measure oxygen routinely in patient tumours. A large-scale collaborative EU-financed project 2009–2014 denoted METOXIA has studied all the mentioned aspects of hypoxia with the aim of selecting potential targets for new hypoxia-specific therapy and develop the first stage of tests for this therapy. A new non-invasive PET-imaging method based on the 2-nitroimidazole [^{18}F]-HX4 was found to be promising in a clinical trial on NSCLC patients. New preclinical models for testing of the metastatic potential of cells were developed, both *in vitro* (2D as well as 3D models) and in mice (orthotopic grafting). Low density

Keywords

Bioreductive compounds, cancer-treatment resistance, carbonic anhydrase inhibitors, dual-activity compounds, non-invasive oxygen detection, targets for new treatment

History

Received 21 August 2014
Revised 15 September 2014
Accepted 15 September 2014
Published online 27 October 2014

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quantitative real-time polymerase chain reaction (qPCR)-based assays were developed measuring multiple hypoxia-responsive markers in parallel to identify tumour hypoxia-related patterns of gene expression. As possible targets for new therapy two main regulatory cascades were prioritised: The hypoxia-inducible-factor (HIF)-regulated cascades operating at moderate to weak hypoxia (<1% O_2), and the unfolded protein response (UPR) activated by endoplasmatic reticulum (ER) stress and operating at more severe hypoxia (<0.2%). The prioritised targets were the HIF-regulated proteins carbonic anhydrase IX (CAIX), the lactate transporter MCT4 and the PERK/eIF2α/ATF4-arm of the UPR. The METOXIA project has developed patented compounds targeting CAIX with a preclinical documented effect. Since hypoxia-specific treatments alone are not curative they will have to be combined with traditional anti-cancer therapy to eradicate the aerobic cancer cell population as well.

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Introduction

For more than 20 years it has been known that there is a correlation between the ability of solid tumours to metastasise and the degree and/or amount of hypoxic tissue in the tumour[1–3]. Since distant metastases represent a major challenge for patient survival after radical and successful treatment of the primary tumour treatment specifically attacking hypoxic cells might have great potential for saving lives. This rationale formed the basis for the call answered by collaborative EU-financed project METOXIA which has been running for 5 years since 1st February 2009. The aim of this large project has been to seek new methods for detection of hypoxic areas in tumours and to study cellular hypoxia-driven responses which could represent new potential targets for the specific killing of hypoxic cells. The most ambitious goal was to take the first steps to develop new cancer treatments based on these targets.

The present research theme raises several challenges. First of all hypoxia is by itself a complicated concept[4,5]. The only common factor for conditions described as hypoxia is that there is less oxygen in the microenvironment than the usual amount in normal tissues. But variability is enormous: In reality hypoxia is a collective or generic term which comprises a whole range of different micro-environmental conditions affecting different molecular regulatory processes in cells. The severity of hypoxia by means of pericellular oxygen concentration is only one aspect. The duration of hypoxia, the sequencing of hypoxic versus aerobic periods (cycling) and separation by slow or quick reoxygenation are others. Production of, as well as protection against reactive oxygen species (ROS) may be pivotal for some of the cellular responses observed. Even oxygenation of normal tissues can vary by a factor of at least eight if different tissues are taken into consideration[6].

Changes in oxygenation activate different regulatory responses in cells, most of these studied by the METOXIA consortium and referred to in this review. Cells are said to be able to sense the change in oxygenation. Stabilisation of the hypoxia-inducible-factor (HIF) protein has been identified as the sensor of hypoxia, activated by very modest reduction in oxygenation. The role of this hypoxia sensing system in cancer therapy is however complicated by lack of cancer-specificity. HIF is usually stabilised by a small reduction of the pericellular oxygen concentration from say, about 4% (which is a normal level in most normal tissues) to about 1% O_2 (i.e. from $\sim$40 to $\sim$10 μM)[7]. Such reduction is readily experienced in various normal tissues under certain conditions, for example in muscle under exercise.

For diagnosis and treatment of cancer some mechanisms activated at lower levels of oxygenation may be more specific for the malignant tissue. It was previously for example customary to consider hypoxia as a level of oxygenation where cellular responses to radiation became significantly reduced compared to the radio-sensitivity under ''normal'' oxygenation. These observations were first noted as early as in the 1930s[8] but were intensely studied in cancer research for many decades after two papers from 1953 and 1955[9,10], which indicated that this radiation protection may be tumour specific since severe hypoxia was tumour-specific.

The radio-resistance of hypoxic cells was later found to be related to the high electron affinity of the oxygen molecule[11], which enables even small amounts of oxygen to fix radiation-induced macromolecular damage before the damaged molecule can be restored by naturally occurring radical scavengers in the cells. So, this is not an active regulation, but rather a chemical process where oxygen reacts more readily than other chemicals to bind radiation-induced radicals. It is worth recalling the great difference in oxygenation between these two extremes: The active regulatory cascades set in motion by HIF-stabilisation at an oxygen level below 1% O_2 and the electron-affinic oxygen-chemical process of fixation of radical damage which was fully counteracted only for oxygen levels below about 0.01%. The level of 1% O_2 was not even considered hypoxia among radiobiologists 50 years ago. It is worth noticing that the correlation between metastasis and tumour hypoxia has been shown in animal models to be just as strong for the different types of hypoxia tested[3]. Thus, the whole range of tumour hypoxia may have a potential for the development of new specific treatment.

In the following review most cell and tissue aspects of hypoxia, therefore, have been included as they all were covered in the METOXIA project, i.e. cell metabolism, cell migratory properties by means of epithelial-mesenchymal transition (EMT), possible new biomarkers for hypoxia imaging and potential individualised treatment, angiogenesis/lymphangigenesis, radio-modifying factors, development of new preclinical models and development of new methods for direct measurement of oxygen plus other micro-environmental factors.

Biological processes regulated by hypoxia

Molecular regulation associated with low oxygen (HIF/Notch/CAIX/pH-regulation)

The presence of tumour hypoxia and/or expression of HIFα in various tumour types have been extensively reported to correlate with increased risk of metastasis[12]. Such associations could explain the involvement of HIF-target genes in biological processes that have an impact on the metastatic spread of cancer cells such as angiogenesis, EMT, cell motility, intra/extravasation and premetastatic niche[13]. In spite of this, we are still far from a comprehensive picture of the hypoxia-driven changes that lead to metastasis formation. With the aim of identifying novel HIF-target genes, we developed a bioinformatics strategy based on metanalysis of gene expression profiles of hypoxic cells combined with phylogenetic foot-printing to identify HIF binding sites[14]. EFNA3, a member of the ephrin type A ligands, was identified as a potential novel HIF target gene with this *in silico* search. Ephrins are cell surface proteins that regulate diverse biological processes by modulating cellular adhesion and repulsion and increasing evidence suggest that ephrin function might be involved in multiple aspects of tumour biology[15]. We were able to demonstrate that hypoxia resulted in increased expression of

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Ephrin-A3 protein through a mechanism involving the HIF-mediated induction of a novel group of lncRNAs encoded by the EFNA3 locus. Although, to our knowledge, this is the first description of such a complex control of gene expression in response to hypoxia, it is likely to be quite prevalent since a recent study demonstrated a profound impact of HIF on the expression of lncRNAs[16]. Indeed, we showed that the stabilisation of HIF within human tumours is associated with increased expression of lncEFNA3 and correlates with incidence of metastasis in breast cancer patients[17]. More importantly, animal models of metastasis revealed a causal link between EFNA3 expression and the induction of metastatic phenotype. Finally, our data demonstrate that Ephrin-A3 expression does not affect primary tumour growth rate or angiogenesis, but instead results in increased ability to intra/extravasate from blood vessels, providing an explanation for the effects on tumour metastatic ability[17]. As these results identify a mechanism by which hypoxia contributes to tumour metastasis, it has also been accepted that enhanced angiogenesis in response to hypoxia is part of an adaptive response aimed at achieving increased oxygen and nutrient delivery to growing tissues. This is mediated by pro-angiogenic factors such as VEGF[18]. On the contrary, it is expected that tumour cells decrease the levels of anti-angiogenic factors in hypoxic conditions in order to favour their own survival and growth. We found that hypoxia diminishes the levels of one such angiogenesis inhibitor, the matricellular protein thrombospondin-1 (TSP-1)[19]. TSP-1 was the first endogenous angiogenesis inhibitor identified[20], and its expression is important for the maintenance of an anti-angiogenic micro-environment; indeed, TSP-1 loss is associated with tumour metastasis and poor outcome[21]. The importance of TSP-1 in maintaining normal kidney angiostasis has been previously demonstrated[22,23]. In addition, our own results have shown that hypoxia-mediated decrease on TSP-1 levels in a renal carcinoma model (ccRCC) was shown to influence cell behaviour enhancing the migratory and invasive potential in *in vitro* assays. However, although most of the hypoxia regulated genes are specifically mediated by HIFs, TSP-1 down-regulation in these tumour cells are not due to a HIF-mediated transcriptional regulation. Instead, Akt signalling and hypoxia-mediated decrease in PHD activity contributes to the down-regulation of TSP-1 in these cells. Therefore, it seems that hypoxia stimulates multiple signals that independently help to decrease TSP-1 levels and these may contribute to the tumour outcome[19].

All these results underline the importance of a precise knowledge of the control of gene expression by hypoxia through HIF-dependent or independent mechanisms in order to obtain a full picture of the cellular adaptations to hypoxia and their impact on the progression of tumours. In this regard, we have recently shown that regulation of gene expression by HIF probably requires its cooperation with a broad set of transcription factors[24]. This cooperation restricts the set of functional HREs among all the available RCGTG motifs within open-chromatin regions. However, it is not the only mechanism contributing to HIF-target specificity and many other genomic features including epigenetic labels and the presence of insulators[25] also restrict the number of genes that are induced in response to hypoxia.

One of the classical HIF-1 targets is carbonic anhydrase IX (CAIX), an enzyme catalysing the reversible hydration of CO_2 and participating in acid–base balance. CAIX is a transmembrane protein expressed in a broad range of solid tumours, but absent from the corresponding normal tissues. Its presence is often associated with poor prognosis and poor response to therapy[26]. Transcription of the CAIX-encoding gene is strongly activated by the HIF-1 transcription factor, which binds immediately upstream of the transcription start site[27]. Hypoxia also induces the activity of CAIX through the PKA-mediated phosphorylation of its intracellular tail[28]. The extracellular enzyme's active site catalyses the conversion of pericellular CO_2 to bicarbonate ions and protons. Bicarbonate ions are transported by bicarbonate transporters across the plasma membrane to the cytoplasm, where they contribute to intracellular neutralisation, which is important for cell survival and proliferation[29]. Protons remain at the outer side of the membrane and support extracellular acidification, which facilitates cell migration and invasion. Accordingly, CAIX is functionally involved in focal adhesion dynamics and in the pH regulating cell migration machinery as part of the spatial and functional complex with the bicarbonate transporters in the lamellipodia of moving cells[30,31]. Thereby, CAIX protects tumour cells from hypoxia and acidosis in the tumour microenvironment and contributes to their invasive and metastatic propensity. Thus, CAIX is not only a surrogate marker of hypoxia and acidosis but also a functional component of the tumour phenotype[29]. This offers opportunities for several anti-cancer strategies based either on immunotherapeutic approaches, or on the enzyme inhibition by the chemical compounds binding to the active site, or on blocking the molecular mechanisms of the enzyme induction and/or activation[32–36].

Mitochondrial reprograming induced by hypoxia

The fine regulation of mitochondrial function has proved to be an essential metabolic adaptation to fluctuations in oxygen availability. During hypoxia, cells activate an anaerobic switch that favours glycolysis and attenuates the mitochondrial activity[37]. This switch involves the HIF-1. We have identified a HIF-1 target gene, the mitochondrial NDUFA4L2 (NADH dehydrogenase [ubiquinone] 1 alpha sub-complex, 4-like 2). Our results, obtained employing NDUFA4L2-silenced cells and NDUFA4L2 knockout murine embryonic fibroblasts, indicate that hypoxia induced NDUFA4L2 attenuates mitochondrial oxygen consumption involving inhibition of Complex I activity, which limits the intracellular ROS production under low-oxygen conditions. Thus, reducing mitochondrial Complex I activity via NDUFA4L2 appears to be an essential element in the mitochondrial reprogramming induced by HIF-1[38].

Cytochrome c oxidase (Complex IV) is the most oxygen-consuming enzyme within the eukaryotic cells and catalyse the transfer of electrons from reduced Cytochrome C to molecular oxygen. We have previously identified NDUFA4 as a novel component of the mitochondrial complex IV[39]. Whereas paralog protein NDUFA4L2 is highly induced by HIF-1 under hypoxia, NDUFA4 protein levels where markedly reduced in hypoxic cells. We demonstrate that Complex IV levels are gradually reduced when oxygen supply becomes limiting. Hypoxia exerts COX sub-unit degradation as clearly evidenced by the diminished expression of NDUFA4 which leads to reduced amount of Cytochrome c oxidase. Upon this condition, relative super-complex organisation is altered, diminishing respirosome and thus becoming more resistant to oxygen deprivation. In addition, hypoxia favours the switch between COX4-1 and COX4-2, which augments relative activity of the individual enzymes. All this data clearly indicate that oxygen tensions regulate the levels of Cytochrome c oxidase[40].

Hypoxia, ER-stress and the UPR

Hypoxia causes ER stress and activation of the UPR

The mechanisms influencing hypoxia tolerance and therapy resistance in tumours are only partially understood[41]. HIF transcription factors promote tolerance through activation of a large number of genes that influence cellular metabolism, pH regulation and

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angiogenesis – phenotypes classically associated with hypoxia. Stabilisation of HIF and activation of its downstream pathways occur at relatively moderate levels of hypoxia (<2% O_2), which is considerably higher than that required to cause radiation resistance (below 0.2%). Work throughout the METOXIA program has shown that more severe hypoxia (<0.2%) leads to rapid activation of the unfolded protein response (UPR)[42–44]. The UPR is an evolutionarily conserved pathway that responds to endoplasmic reticulum (ER) stress by the activation of three ER stress sensors present within the ER membrane, PERK (EIF2AK3), inositol requiring kinase 1 (IRE1/ERN1), and activating transcription factor 6 (ATF60)[45]. They are activated through a common mechanism involving sequestration of BIP (HSPA5) by misfolded protein from the luminal domains of the sensors. Upon activation, ATF6 translocates to the Golgi apparatus and is cleaved to release an active transcription factor, while the kinase/endoribonuclease IRE-1 excises an intron from the XBP-1 transcription factor pre-mRNA. ATF6 and XBP1 induce ER chaperones and proteins involved in ER protein maturation. PERK phosphorylates the serine 51 residue of eukaryotic initiation factor 2α (eIF2α). This event inhibits translation at the initiation step, and mitigates ER stress by reducing additional ER protein load. In addition, eIF2α phosphorylation redirects translation towards a sub-set of transcripts, including the transcription factor ATF4[46]. ATF4 induces a large number of genes, including the transcription factor CHOP. CHOP in turn induces GADD34, which directs phosphatase activity against eIF2α, setting up a negative feedback loop to fine-tune signalling through this pathway. We have shown that hypoxia causes activation of the UPR, including all its three arms[42,47]. Activation of PERK signalling occurs within 30 min of hypoxia, and is capable of responding to rapid changes in oxygenation typical of those that occur during cyclic hypoxia in tumours.

Hypoxia induces ER stress due to defects in disulphide bond formation

The UPR is a stress response primarily tailored to alleviate proteotoxic stress originating from the ER due to the presence of unfolded or misfolded proteins in this organelle. The ER serves as the first maturation compartment for proteins destined for the extracellular space, and is home to N-linked glycosylation and disulphide bond formation, as well as further glycan processing and disulphide bond rearrangements (isomerisation), all accompanied by protein folding. Unsuccessful protein folding leads to exposed hydrophobic domains that sequester chaperones and activate the UPR which regulates translation and transcription in concert to prevent further ER cargo load and to increase ER capacity. Rapid activation of the UPR during hypoxia suggested that the ER is directly sensitive to oxygen levels, potentially resulting in impaired protein folding. Work within METOXIA has led to an understanding of the mechanistic basis for hypoxia-induced ER stress and UPR activation, and revealed a novel requirement for oxygen in protein folding[47]. Proper folding of proteins that mature in the ER often requires formation of disulphide bonds, which are introduced both co- and post-translationally by protein disulphide isomerase (PDI)(P4HB) and family members in a redox reaction where disulphide bonds within PDI's CXXC active site are reduced[48]. PDI must subsequently be reoxidised, a reaction catalysed by the ER oxidases Ero1α (ERO1L) and Ero1β (ERO1LB). The ERO1-bound FAD can be reoxidised by molecular oxygen in a reaction that produces stoichiometric hydrogen peroxide (H_2O_2)[49]. Recently, we demonstrated the existence of two distinct phases of disulphide bond formation in living cells with differing requirements for oxygen[47]. We showed that co-translational disulphide bond formation occurs normally without oxygen, indicating the existence of alternative oxidants under hypoxic conditions. However, post-translational disulphide rearrangement (isomerisation) is completely dependent on oxygen. Consequently, proteins that do not require disulphide isomerisation are expressed normally even under anoxia, whereas those that require disulphide isomerisation remain unfolded in the ER. Immature ER cargo proteins remain reversibly trapped in the ER during hypoxia to variable extents, perhaps depending on the complexity and/or fidelity of disulphide bond formation and rearrangement. This effect likely represents the source of ER stress during hypoxia, since the dependency on oxygen closely mirrors that of UPR activation, and a disulphide-lacking protein was processed and transported normally through the secretory pathway in the absence of oxygen.

The UPR promotes hypoxia tolerance through autophagy and ROS detoxification

Activation of the UPR, contributes to hypoxia tolerance through supporting pro-survival and detoxification mechanisms. Several reports indicate that hypoxia itself is a very powerful trigger for the induction of autophagy, a pro-survival pathway that allows recycling of cellular constituents. Hence, autophagy is primarily localised in the hypoxic fraction of the tumour[50–52]. The widely used and accepted autophagy marker, LC3b[53], is partly integrated within the autophagosome causing partial degradation during autophagy. Turnover of LC3b can therefore be used as a measure for the rate of autophagy or autophagic flux. Besides its use in determining autophagy activity, LC3b is crucial in autophagy execution. LC3b coats the extending membrane allowing it to fuse and create the autophagosome. Hence, LC3b deficiency leads to impaired autophagy and increased cell death[54,55]. During hypoxia, autophagy is rapidly activated and induces a high autophagic flux[50]. The degraded LC3b requires replenishment in order to maintain the high autophagic flux. This is mediated through activation of the ''PERK-arm'' of the UPR. Activation of PERK leads to expression of the transcription factor, ATF4, that directly binds the LC3b-promoter, and causes transcriptional up-regulation of LC3b[50,56] and an important autophagy activator, ULK1[57,58]. Correspondingly, cells deficient in PERK-signalling fail to transcriptionally induce LC3b and become rapidly depleted of LC3b protein during hypoxia and thus fail to maintain the autophagic process.

In the context of cycling hypoxia, cells experience elevated levels of ROS-production[59]. The PERK-arm of the UPR is required for direct mitigation of ROS through ATF4-dependent transcription. After PERK-activation, ATF4 transcriptionally upregulates sub-units of the cysteine/glutamate antiporter xCT (SLC7A11) and 4F2hc (SLC3A2), the glycine transporter GLYT1 (SLC6A9), CTH (Gysthathione gamma-lyase), and GCLC[46,60,61]. Both cysteine and glycine are required for glutathione synthesis, where cystathionine γ-lyase can also contribute by converting cystathionine to cysteine. Furthermore, GCLC is a rate-limiting enzyme in production of gluthathione. Hence cells deficient in PERK signalling and therefore ATF4 expression display compromised cysteine-uptake, reduced glutathione production and are exposed to elevated levels of ROS. The decreased glutathione levels result in cancer cells that are sensitive to oxidative stress[61].

Both for chronic and acute hypoxic cells, PERK-signalling is important for adaptation and response to stress. Consequently, cells deficient in PERK-signalling display lower hypoxia tolerance and increased cell death *in vitro*. Tumours in which PERK-signalling is targeted have a reduced fraction of hypoxic cells and are sensitised to irradiation[50,61].

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The UPR regulates metastasis

Tumour hypoxia has also been recognised as an important contributor to the distant metastasis of several human cancers, including cervix[62]. Likewise, *in vitro* exposure of cancer cell lines to hypoxia increases pulmonary metastasis[1] and increasing tumour hypoxia *in vivo* increases metastasis in xenograft models[63]. Experimental studies aimed at elucidating the signalling events underlying hypoxia-induced metastasis have largely focused on the HIF1 pathway. However, we have demonstrated that hypoxic activation of the UPR also drives the metastatic phenotype in an orthotopic animal model of human cervical carcinoma[64]. Previous studies using this model have shown that subjecting animals carrying primary cervix tumours to conditions of cyclic hypoxia directly enhances metastatic spread to the local lymph nodes. We have shown that interruption of signalling through the PERK/eIF2α/ATF4-arm of the UPR in established tumours results in complete inhibition of hypoxia-driven lymph node metastasis. The changes in metastatic capacity were the result of reduced cell survival during hypoxia following disruption of the UPR. However, we also found that the PERK/ATF4 target gene LAMP3[65], a metastasis-associated gene, is a key mediator of hypoxia-driven lymph node metastasis. Silencing LAMP3 expression significantly inhibited lymph node metastasis in response to hypoxia, but did not affect hypoxia tolerance or tumour growth. Instead, we found evidence for a role of LAMP3 in regulating hypoxia-induced cell migration. We also investigated LAMP3 expression in human cervix tumours, one of the cancer types in which hypoxia is known to stimulate metastasis, and demonstrated that LAMP3 is regulated by both amplification as well as by hypoxia. These findings suggest that the poor prognosis of patients with hypoxic cervix cancer is due in part to PERK/eIF2α/ATF4 activation of LAMP3 and increased metastatic capacity.

The UPR as a target for therapy

All three arms of UPR have been shown to contribute to cancer cell survival while PERK and IRE1α have been the most studied and most promising as pharmacological targets. IRE1 is a highly conserved signalling arm of the UPR and, sequencing of cancer genomes revealed IRE1α[66] and its target, XBP1[67], to have higher frequency of mutations in cancer. Cells deficient in XBP1 have decreased hypoxic cell survival *in vitro* and a delay in tumour growth *in vivo*[68]. Similarly, our group has shown that IRE1α knockdown cells have impaired proliferation under hypoxia[69]. However, the PERK arm of UPR appears to have the most significant role in tumour hypoxia tolerance and survival. This may be attributed to PERK's ability to transcriptionally (e.g. ATF4) and translationally (eIF2α phosphorylation) control genes essential in cell survival and homeostasis. The importance of the UPR during hypoxia, and in the survival of cancer cells subjected to ER stress, has stimulated interest from industry to develop inhibitors against both PERK[70,71] and IRE1[72,73] pathways. Pharmacologic targeting of IRE1α signalling exclusively focused on either its RNase domain or the ATP-binding pocket. Such RNase domain inhibitors include STF-083010[74] and 4 μ8C[72] prevent splicing of the intron in XBP1 mRNA. APY29 or sunitinib inhibit the ATP-binding pocket but allosterically activate the RNase[75], while compound **3** (Figure 5) is an inhibitor of both active sites[76]. In pre-clinical *in vivo* studies, STF-083010 slowed the growth of human multiple myeloma xenografts[74] proving potential for use of IRE1 inhibitors for the treatment of this cancer. The PERK pathway has been targeted either through inhibition of the PERK kinase[77], or its target eIF2α[78]. The optimised and orally active PERK inhibitors, GSK2656157, inhibited growth of a number of pancreatic and multiple myeloma tumour xenografts[70]. Our group utilised potent small-molecule inhibitors of IRE1α endonuclease activity, 4 μ8c[72] and PERK kinase activity, GSK compound 39[77], and compared the therapeutic potential of targeting these two different arms of the UPR. Surprisingly, we found that selective and potent inhibition of IRE1 splicing activity had no effect on cell proliferation or survival of cells exposed to hypoxia. This was in contrast to inactivation of PERK, which, like the genetic models[50,61] substantially reduced tolerance to hypoxia and other ER stress-inducing agents[50]. The success of PERK inhibitors and the discovery of important downstream survival pathways regulated by PERK during ER stress such as, autophagy, anti-oxidant system and protein folding, have placed PERK at the forefront of potential UPR therapeutic targets in cancer.

The role of ROS in hypoxia

Superoxide and other ROS have been previously related to oxidative stress conditions, leading to the damage of cellular proteins, RNA, DNA, and lipids, and subsequently to the pathology of different diseases including tumour initiation and progression.

In recent years, superoxide and other ROS have been acknowledged to act as important signalling molecules in various physiological and pathophysiological conditions. Since superoxide is derived from molecular oxygen, ROS and ROS-dependent signalling appear to be connected in different ways to the pathways involved in the adaptation towards hypoxia.

One of the major pathways regulated by oxygen availability relies on the activity of HIF. Originally described to be only induced and activated under hypoxia, accumulating evidence suggests that HIFs play a more general role in the response to diverse cellular activators and stressors, many of which use ROS as signal transducers. On the other hand, the HIF pathway has also been implicated in controlling some ROS-generating systems such as NADPH oxidases. Thus, an important cross-talk exists between ROS signalling systems and the HIF pathway which appears to have implications for the pathogenesis of various disorders including cancer.

ROS and antioxidants

Superoxide anion radicals ($O_2^{-\bullet}$) are formed from molecular oxygen by acquisition of an electron and can further react to other ROS such as hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^\bullet$), peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl) and singlet oxygen ($^1O_2$). $O_2^{-\bullet}$ is not freely diffusible, but can cross membranes via ion channels[79,80]. H_2O_2 on the other hand, which is not a radical, is diffusible and has therefore been frequently considered to act as second messenger. In the presence of iron, superoxide and hydrogen peroxide can lead to the formation of highly reactive hydroxyl radicals which can damage cellular proteins, RNA, DNA, and lipids. Interaction of ROS with nitric oxide (NO) or fatty acids can lead to the formation of peroxynitrite or peroxyl radicals, respectively, which are also highly reactive[81].

Antioxidant enzymes and antioxidant scavengers contribute to control the levels of ROS and to prevent oxidative stress reactions. The nuclear transcription factor Nrf2 has been considered to play an important role in regulating gene expression of antioxidant enzymes[82]. Among the most prevalent antioxidant systems are superoxide dismutases (Cu/Zn SOD, EC SOD, and Mn SOD), catalase, glutathione peroxidases (GPX), thioredoxins (TRX) and peroxiredoxins (Prxs)[83]. Antioxidant scavengers are predominantly of dietary origin. These biomolecules include tocopherol, ascorbic acid, carotenoids, uric acid, and polyphenols.

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NADPH oxidases generate superoxide

Among the enzymatic systems which have been shown to be able to release ROS, NADPH oxidases are unique in that their sole function is to generate ROS. NADPH oxidases have been identified in leukocytes as part of the innate immune response. Subsequently, NADPH oxidases have been identified in many non-phagocytic cells including vascular cells and tumour cells (for review refer: Gorlach et al.[84] and Bedard & Krause[85]). NADPH oxidases are multi-protein enzymes which transfer an electron from NADPH to oxygen to generate $O_2^{-\bullet}$. Major components of the NADPH oxidases are transmembrane NOX proteins. To date, 5 homologous NOX proteins termed NOX1 to NOX5 have been identified. Apart from NOX5, all NOX proteins form together with the protein p22phox a cyctochrome b558[86,87].

The complex composition of the different NADPH oxidases allows them to respond to a variety of stimuli such as growth factors, cytokines, hormones, vasoactive factors and coagulation factors mainly via receptor-operated signalling pathways[88]. In addition, NADPH oxidase activity can also be regulated at the level of expression, whereby transcriptional mechanisms seem to be the most prevalent pathways. This allows NADPH oxidases to respond also to changes in micro-environmental conditions including variations in oxygen availability.

Reactive oxygen species modulate hypoxia-inducible factors

Early evidence indicated that the hypoxia-inducible transcription factor HIF-1 is redox sensitive since treatment of purified HIF-1 with H_2O_2, diamide or N-ethyl-maleimide prevented the ability to bind DNA under hypoxic conditions, while dithiothreitol could preserve DNA binding, suggesting that HIF-1 DNA binding requires reducing conditions[89]. Trx has been shown to enhance HIF-1α protein levels due to interaction of the Trx effector Ref-1 with the HIF-1α TADN and TADC, a reaction which seemed to be dependent on the redox state of cysteine 800 in HIF-1α and cysteine 848 in HIF-2α[90,91]. Mutation of cysteine 800 prevented the decrease in HIF-1α TADC activity in response to hydroxyl radicals ($OH^{\bullet}$)[92] supporting the notion that a reducing environment is preferential for stabilisation and functioning of HIF-α proteins.

In contrast, while administration of external H_2O_2 or expression of MnSOD seemed to prevent hypoxic induction of HIF-1α in tumour cells[90,93] application of low concentrations of H_2O_2 under normoxic conditions or overexpression of Cu/ZnSOD or MnSOD increased the levels of HIF-1α in vascular cells[94–97] but also in Hep3B cells[98]. Subsequent evidence was provided that HIF-α proteins are responsive to a variety of non-hypoxic stimuli in a ROS-dependent manner, including, insulin[99], growth factors[97,100,101], thrombin[97], angiotensin-II[101], peptides[102], TNF-alpha and cytokines[103,104], the ''hypoxic mimetic'' $CoCl_2$[94,98] and various other stressors. Several studies identified NADPH oxidases as important sources of ROS in the regulation of HIF-α in vascular cells[95–97,102,105,106]. NOX4 was shown to control HIF-1α and HIF-2α levels in smooth muscle cells[95,96]. NOX2 and NOX5 which are important for endothelial ROS generation[86] also play a role in the regulation of HIF-1α[102,106] in these cells.

NADPH oxidases and HIF in tumour cells

Since NADPH oxidases have been shown to up-regulate angiogenic factors known to be controlled by HIF such as VEGF or PAI-1 and to promote angiogenesis[102,107]. ROS generation by NADPH oxidases might also contribute to tumour angiogenesis. In fact, xenografts deficient in NADPH oxidase activity had reduced vascularisation (Görlach et al. unpublished observation). In support, increasing evidence suggests that NADPH oxidases are also expressed in tumour cells and are important regulators of tumour growth and progression[108]. Since HIF transcription factors play a central role in tumour biology, a cross-talk between NADPH oxidases and HIFs may be important also in tumour biology. In line with this assumption, tumour treatment with hyperthermia was shown to enhance NOX1 and subsequently HIF-1α levels in tumour cells[109]. NOX1 was also shown to increase HIF-1α levels in lung tumour cells[110] while NOX4 knockdown decreased HIF-1α levels in ovarian cancer cells[111]. Depletion of NOX4 or NOX1 reduced HIF-2α levels in VHL-deficient 786-O or RCC4 renal carcinoma cells suggesting that ROS may act via a VHL-dependent pathway[112,113]. Interestingly, p22phox was shown to maintain HIF-2α protein levels through inactivation of tuberin and downstream activation of ribosomal protein S6 kinase 1/4E-BP1 pathway[114], and to promote xenograft growth (Görlach et al. unpublished observation). These data indicate that NADPH oxidases are important sources of ROS in a variety of non-hypoxic pathways also in tumour cells.

Mechanisms of HIF regulation by reactive oxygen species and NADPH oxidases

ROS have been shown to regulate HIF-α levels by different mechanisms. H_2O_2 application or NOX4 overexpression increased HIF-2α TADN and TADC activity and this response was abolished upon mutation of the target prolines or asparagines, respectively[96]. Similar observations were made with HIF-1α indicating that ROS can affect HIF-α stability by interfering with the PHD/pVHL pathway. In *junD*-deficient cells, ROS interfered with Fe(II) availability in the HIF prolyl hydroxylase catalytic site possibly by a Fenton-type reaction thus diminishing HIF-1α hydroxylation and allowing its accumulation[115]. Similarly, NOX4-dependent ROS generation decreased the availability of Fe(II) thereby increasing HIF-1α levels[96]. Addition of iron, on the contrary, increased HIF-1α degradation[116] indicating that the balance between Fe(II) and Fe(III) is of major importance in controlling HIF-α levels. In this context, ascorbate seems to ac via maintaining Fe(II) levels in the cell thereby controlling PHD/FIH hydroxylase activity[117]. Subsequently, provision with ascorbate decreases HIF-α levels under non-hypoxic conditions[94,96,97,116,118]. On the other hand, thrombin and angiotensin-II decreased cellular ascorbate levels while increasing HIF-α levels[96,118] further suggesting that ascorbate availability may provide an important mechanism in the regulation of HIF-α under non-hypoxic conditions. Similarly, other reducing agents such as glutathione and dithiothreitol can promote HIF hydroxylase activity further indicating that the cellular redox state is important in controlling PHD activity[119]. Mutation of a previously recognised redox-sensitive cysteine in PHD2[120] increased basal hydroxylation rates and conferred resistance to oxidative damage *in vitro*, suggesting that this surface-accessible PHD2 cysteine residue is a target of anti-oxidative protection by vitamin C and glutathione[121].

In addition to regulation of HIF-α at the level of protein stability induction of HIF-1α has been described to be regulated by a transcriptional mechanism in a ROS-dependent manner (for review, refer Gorlach, 2009[122]). Subsequently, it was shown that HIF-1α is a direct target gene of NFκB[95,123–127], and that ROS derived from NADPH oxidases or direct application of H_2O_2 regulate NFκB-dependent HIF-1α transcription [95,106,128]. These findings indicate that transcriptional regulation of HIF-1α by ROS-sensitive activation of NFκB may represent an important mechanism how agonists can induce HIF-1α under non-hypoxic conditions and provide a pathophysiologically interesting link between these two important redox-sensitive transcription factors

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with various implications not only for inflammatory conditions, but also for cancer.

In contrast to HIF-1α, only limited data are available on the regulation of HIF-2α mRNA and the contribution of ROS. It has been shown that NOX4 depletion decreased HIF-2α mRNA levels in RCC4 cells[113]although the underlying mechanisms have not been elucidated. Since the NFκB binding site which mediates transcriptional regulation of HIF-1α does not seem to be conserved in the HIF-2α promoter, this may be an important factor determining non-redundant functions of HIF-1α and HIF-2α in hypoxic and non-hypoxic conditions. In hematopoietic stem cells stimulated with erythropoietin, HIF-2α was identified as a direct Stat5 target gene. Although not explicitly studied, this mechanism may also involve ROS[129].

Subsequently, HIF-1α protein synthesis has been considered to be regulated by cap-dependent signalling processes, mediated mainly through PI3K/Akt and mTOR in particular in response to tyrosine kinase signalling[130,131]. ROS derived from NADPH oxidases and mitochondria have been shown to be able to activate PI3K/Akt signalling in normal and malignant cells[81,97,132,133] and have been implicated in translational regulation of HIF-1α in smooth muscle cells in response to angiotensin-II[134]. In addition it was proposed that the PI3K pathway in conjunction with NFκB may be involved in the translational regulation of HIF-1α in response to TNF-α[135]. Recently, it was shown that HIF-2α mRNA translation was controlled by p22phox by a mechanism involving stabilisation of Rictor-associated mTORC2 complex in the absence of VHL through an eIF4E-dependent mRNA-translational mechanism[136]. However, the relative importance of ROS-dependent HIF-α translational compared to transcriptional mechanisms and protein stabilisation has not been clarified, yet.

NADPH oxidases in the hypoxic environment

NADPH oxidases have been shown to be relevant in situations of ischaemia/reperfusion or cyclic hypoxia. In models of stroke or myocardial infarction, as well as in intermittent hypoxia simulating sleep apnoe NADPH oxidases have been described to contribute to increased ROS levels[137–140]. In lung tumour cells NOX1 was shown to contribute to up-regulation of HIF-1α in response to intermittent hypoxia[141]. NOX4 was shown to contribute not only to ROS generation in response to cyclic hypoxia in different brain tumours, but also to tumour progression and radio-resistance under these conditions, similar to HIF-1α[142]. In hindlimb ischaemia, NOX2 mediated HIF-1α regulation in the bone marrow[143]. In addition, NOX2 was also shown to contribute to enhanced HIF-1α levels in the carotid body and in PC cells in response to intermittent hypoxia (for review, refer Prabhakar & Semenza[144]).

In many cases, these observations were accompanied by increased levels of NADPH oxidase sub-units including p22phox, p47phox and different NOX proteins. Since it has been shown that several NADPH oxidase sub-units can be induced by ROS, including p22phox, NOX2, NOX4 and Rac1[102,107,145] increased ROS levels in the reoxygenation/reperfusion periods may be responsible for such an up-regulation thereby promoting sustained ROS generation under these conditions.

In addition, NADPH oxidases have also been shown to play a role in the response to sustained hypoxia. NOX2−/− mice were protected against the development of hypoxia-induced pulmonary hypertension[146], and this effect was concomitant with decreased ROS levels. Similarly, hypoxia further decreased $O_2^{-\bullet}$ release in p47phox-deficient perfused lungs[147] indicating that NADPH oxidases contribute to ROS generation under hypoxia.

In fact, hypoxia can induce the levels of several NADPH oxidase sub-units, including NOX4[148,149]. Induction of NOX4 by hypoxia helps to maintain ROS generation under hypoxia, and is responsible for increased ROS generation upon reoxygenation in vascular cells[148]. Interestingly, hypoxic induction of NOX4 has been shown to contribute to hypoxic HIF1α upregulation in different cell types similar to the situation under normoxia[148,150]. Conversely, NOX4 was identified a genuine HIF-1α target gene[148]. Functional hypoxia response elements were also identified in the human NOX2 and Rac1 promoters indicating that NADPH oxidases as genuine HIF target genes are involved in the adaptive response to hypoxia[102,105]. Experiments in mice deficient in endothelial HIF-1α confirmed the relevance of this transcription factor in the regulation of NADPH oxidases[102]. Although the exact importance of hypoxia and HIF-dependent up-regulation of NADPH oxidases is still not clarified, one may speculate that a certain level of ROS is important for maintaining basal cellular functions under oxygen-limited conditions and may thus help to protect against apoptosis or cell death at least at the cellular level. Furthermore, in leukocytes and other immune cells, induction of NADPH oxidases under hypoxic conditions may contribute to initiation and propagation of inflammatory conditions frequently associated with conditions of oxygen deficiency. This would also explain the protective effects of NADPH oxidase deficiency seen in different animal models of intermittent and sustained hypoxia. Moreover, recent data also indicate that NADPH oxidases might be associated with outcome to tumour therapy since they can modulate the DNA damage response (Görlach et al. unpublished observation).

In essence, there is a tight cross-talk between hypoxia and HIF signalling on the one hand, and ROS and NADPH oxidases, on the other hand, which allows tight control of HIF activity dependent on the current redox state. Since fluctuations in the redox state are commonly observed in the changing tumour environment, this cross-talk might be of particular importance in the adaptive response of tumour cells to their environment with important consequences for therapeutic sensitivity.

Technology

Standardisation of *in vitro* cell microenvironments

Cell culture monitoring with microsensors provides insight into the cellular metabolism as well as regulatory pathways by continuous measurements using sensors for small molecules and biosensors. The knowledge of the pericellular values of typical metabolic parameters such as dissolved oxygen, pH value, glucose and lactate is essential for standardisation of cell culture experiments. Furthermore, these parameters form the basic set of variables for control of *in vitro* experiments (see next Section: ''Microphysiometry for drug testing and cancer research''). In 2007, we introduced the concept of the Sensing Cell Culture Flask (SCCF)[151,152] providing a technological platform for cell culture monitoring without the need to deviate from tissue culture flasks as the conventional format for cell culturing (Figure 1).

The SCCF system features a microsensor chip embedded in the bottom of a culture flask. Thus, pericellular parameters can be monitored from cells directly settling on the sensor chip. The chip itself is transparent allowing optical inspection with common phase contrast microscopes. The SCCF platform allows the integration of ideally any electrochemical or biosensor by simple adjustments during the post-processing steps of the chip fabrication. Oxygen sensors are based on platinum working electrodes covered with hydrogel acting as a diffusion limiting membrane, which were operated by chronoamperometric protocols[153]. An example for potentiometric sensors is the pH sensor using iridium oxide electrodes as the ion-sensitive material. Biosensors for glucose and lactate have been integrated by dispensing from a

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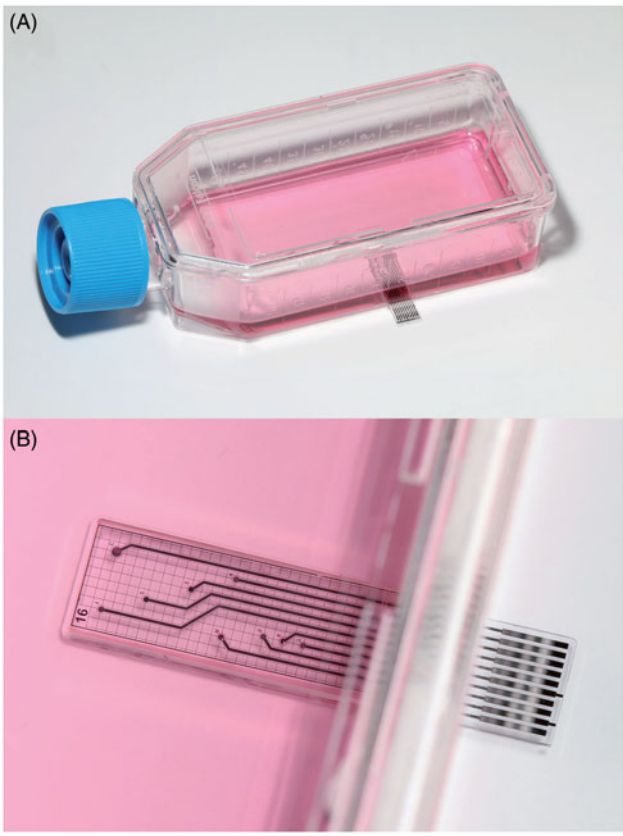

Figure 1. Sensing cell culture flask (SCCF), a microsensor chip is embedded in the bottom of a conventional tissue culture flask (A), detailed view of the transparent microsensor chip (B) with its electrical contact pads outside the flask.

two-layer stack of hydrogels consisting of the enzyme membrane and a diffusion limiting membrane onto platinum electrodes[154]. The enzymes used have been glucose, respectively, lactate oxidase, which convert the analyte to hydrogen peroxide, which can be subsequently oxidised at the platinum electrode. The flexibility of the SCCF technology concept also allows the integration of a specific sensor for other small molecules beyond basic metabolic parameters. The most recent enhancement of the system was the integration of sensors for superoxide.

The production of ROS, especially superoxide anions, is a common attribute of all aerobic cells. Intrinsic ROS generation is mainly linked to the mitochondrial respiration chain, whereas superoxide is produced as by-product during aerobic respiration. Other sources may be the activation of oxidoreductase enzymes and metal catalysed oxidation. The disruption of cell redox homeostasis leads to oxidative stress by decreasing ROS scavenging ability or by increased ROS production. Due to their high energy demand, cancer cells often show increased ROS production, whereas these cells are able to adapt to higher oxidative stress with the consequence of inhibited apoptosis, promoted malignant transformation and metastasis, as well as resistance to anti-cancer drugs[155,156]. Interestingly, the cell and probe handling as well as cell culture condition may attribute to measured endogenous ROS production signals[157].

The measurement of superoxide levels in cell culture is often conducted by fluorescence spectroscopy or electron paramagnetic resonance (EPR) with the drawback of extensive cell culture treatment, the absence of continuous long-term monitoring ability and often selectivity issues[158]. Electrochemical superoxide detection by direct oxidation on modified gold electrodes offers the possibility for selective monitoring of ROS levels during *in vitro* cultivation. The permanent mounting of sensors directly in the culture area allows a real-time detection of ROS in the direct extracellular microenvironment without disturbing cell culture routines. The measurement principle is based on direct amperometric oxidation of superoxide anions on gold electrodes. A low oxidation potential as well as an appropriate sensor coating with a polymer layer assure a selective and sensitive radical monitoring during acute experiments.

Microphysiometry for drug testing and cancer research

In microphysiometry systems, cells are cultured on a chip, and metabolic parameters are measured non-invasively. In contrast to the SCCF (see Section ''Standardisation of *in vitro* cell microenvironments''), medium is exchanged periodically in a stop/flow cycle. After determination of reference metabolic rates, substances can be added to the medium in order to alter the metabolism. Metabolic products and cellular behaviour upon substance exposure are then measured by the sensors. In contrast to end-point analysis, these systems allow continuous online monitoring, such as the study of pharmacodynamics and recovery effects. The measured parameters typically include extracellular acidification due to the excretion of protons and cellular adhesion to the surface. Other important parameters include oxygen consumption due to cellular respiration, or the energy metabolism, primarily glucose uptake and lactate production.

A number of different microphysiometers have been developed, most of them based on silicon chips, lacking the optical transparency for phase contrast microscopy. The Cytosensor included a light addressable potentiometric sensor (LAPS) on a silicon chip to measure pH[159–161]. It was commercialised by Molecular Devices, and a number of modifications were developed. Amperometric biosensors were included to measure glucose and lactate[162,163].

Cellular adhesion and morphology was measured with interdigital electrode structures (IDES), based on impedance[164]. Oxygen- and pH-monitoring was added by ion selective field effect transistors (ISFET) or amperometric oxygen sensing on noble metal electrodes[165–168]. The Bionas Discovery 2500 system featured IDES for adhesion and ISFETs for pH measurement and included palladium electrodes as amperometric oxygen sensors[169,170]. These systems were applied in pharmacological and toxicological studies or environmental monitoring.

Since optical transparency is a much needed feature, we have developed a system based on a glass chip to allow phase contrast microscopy[171]. The basic technology and the surface on which the cells grow are shared with the SCCF. It includes electrochemical microsensors for oxygen, pH, glucose and lactate. Oxygen is measured amperometrically with platinum electrodes; pH is measured potentiometrically with iridium oxide electrodes. The medium is supplied by efficient, low volume microfluidics. The enzyme-based biosensors for glucose and lactate are integrated in the microfluidics. That allows the biosensors to be placed outside of the cell culture area to enhance biocompatibility, because the exposure of the cells to the hydrogen peroxide produced by the enzymes can be avoided.

The dimensions of the system are designed to fit the pitch of a 24-well micro-titre plate, with all sensors fully integrated on-chip (Figure 2). In a first phase, cells are cultured on the glass chip at the bottom of the well for up to several days. Then, in the measurement phase, the well is sealed to form a gas-tight microfluidic system with only 10 µl total volume. The medium now needs to be exchanged periodically with a pump in stop/flow cycles. Because the medium layer above the cells is drastically reduced to only ~100 µm, the cells' metabolism alters the medium quickly.

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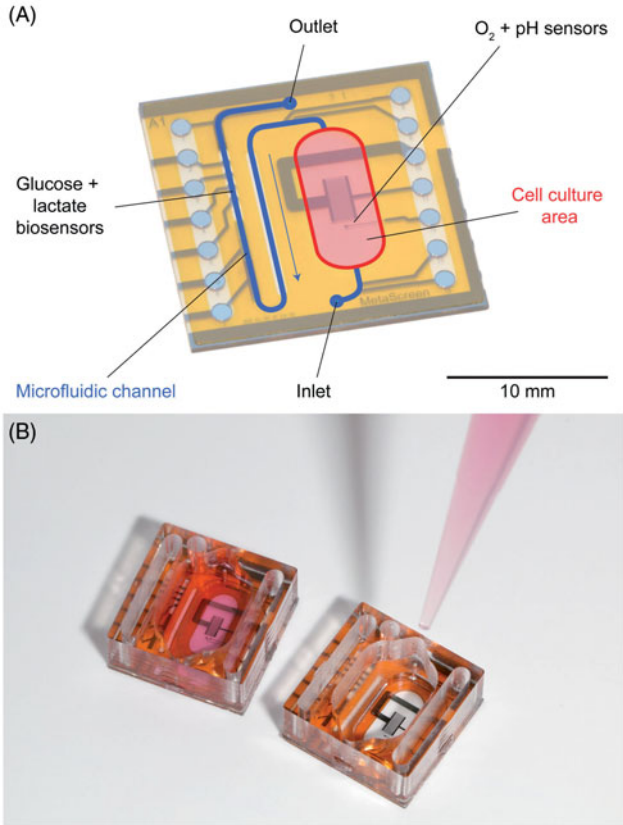

Figure 2. Microphysiometry chip (A). Oxygen and pH are measured in the cell culture area during the stop phase. Glucose and lactate are measured during the flow phase in the outlet microfluidic channel. Photography of microphysiometry chip with cell culture well filled for cell culturing phase (B).

This principle gives access to cellular metabolism and measurable changes in microenvironment within a few minutes, much faster than in a large flask. Over the course of a few hours, reference metabolic rates can be determined and then altered by the addition of substance. Measurement takes place both during the stop phase, where oxygen and pH are measured in the cell chamber, and when the used medium is flushed to the biosensors in the outlet channel.

T98G human glioblastoma and T-47D breast cancer cells were cultured in the system for at least 24 h before measurements. Cell morphology can be observed at any given point due to the system's transparency. In stop/flow phases of around 5 min each, an immediate oxygen consumption and acidification was observed. In the outlet, the amount of consumed glucose and the produced lactate was quantified. The fast and efficient medium exchange by microfluidics was demonstrated. Over several hours, stable rates in a large number of cycles could be determined. These rates were altered by the addition of drugs, e.g. cytochalasin B, upon which an immediate change in cellular metabolism was determined. After supply of regular medium, the recovery of the metabolism could be observed, demonstrating the advantage of continuous monitoring in comparison to end-point tests. A low total volume per cycle of only 15 µl was achieved, reducing the consumption if the drug is available only in limited amounts.

We developed a new, transparent microphysiometry platform, including the integration of glucose and lactate biosensors and low volume microfluidics. The system allows the precise, continuous monitoring of tumour cell metabolism and the assessment of metabolic response to drug exposure.

Automation of cell culture: From routine to intelligent systems

Cell culture techniques are typically used to produce (therapeutic) antibodies and artificial tissues, provide cell material for, for example toxicity and other cell-based assays, in which mammalian cells are used as a ''physiological barometer of the drug/target interaction in HTS and early clinical profiling''[172].

Manual cell culture is a process that requires many hours of repetitive, painstaking operations. Furthermore, living cells require maintenance of a favourable microenvironment throughout each passage including cell growth, harvesting, reseeding, and analysis[173]. From this point of view it is obvious, that the development of automation equipment for these repetitive steps should be a key issue for the future. This review section will give an overview of the current state of ''cell nursing'' in medical biotechnology with the focus on possibilities for automation.

In Germany, for instance, more than 200 biotechnology companies are working on the development of new therapies and diagnostics, thus representing the commercial successful, medical section of biotechnology, the so-called ''red'' biotechnology. And in this field cancer represents one of the most commonly researched diseases.

Automated high throughput cell cultivation: fully automated cell culture maintenance

In red biotechnology the highest level in automation can be found traditionally in high-throughput screening (HTS), a technology used in the early stages of drug discovery. Approximately half of the HTS assays performed are cell-based assays. That means, culturing of large amounts of mammalian cells, which are dispensed into small volumes, e.g. the mainly used multi-well micro-titre plates. This is all done in a highly standardised way, because the cells are the main ''reagent'' for the subsequent cell-based assays. As in manual cell culturing, the control of environmental factors is of importance and sub-cultering conditions should reliably be kept constant (relative humidity R_H at 95%, 5% CO_2 and a temperature of 37 °C). Robotic incubators are, beside the incubator itself, additionally equipped with tools and devices for transporting, storing and handling vessels, flasks, dishes, and bottles. Additionally media pumps and dispensing devices are needed for pipetting of media and suspended cells. The whole automatic cell culture platform is housed in hood/hoods equipped with HEPA filters[174].

The ''gold'' standard of HTS robots is the SelecT (Sartorius, former TAP) developed for cultivation of adherent cells in flask, also in triple and HYPERflasks. A robotic arm, operating in six axes, is able to operate like a human arm. Culturing adherent cells, comprises the following processes: seeding, feeding the cells by replacing exhausted media with fresh media, and the most critical step, passaging of the cells. Passaging attached cells involves detaching enzymatically (trypsinisation) a confluent cell layer from the bottom of the vessel in several incubation and washing steps. The suspended cells are then transferred into larger or multiple vessels for further cultivation. Up to 20 different cell lines can be grown in parallel in maximal 182 flasks, all managed by the software, which also controls up to 15 media pumps. Optionally a multi-format cell dispenser for 96, 384 and 1536-well plates situated in a separate hood can be added[172,173,175].

Both, the Freedom Evo from Tecan[176] and the Star system from Hamilton[177] are based on pipetting robots. The STAR pipetting robot is designed to feed and passage adherent cells grown in multi-well plates. For cells in suspension, which need to be shaken, a small cell culture incubator add-on is available, that fits inside the STAR hood.

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In an impressive example for an extended automated cell culture protocol is given[177]. Confluence and trypsinisation-time can be entered through a graphical user interface GUI. The system checks the actual confluence by means of an automated microscopic cell analyser platform (Cellavista, Roche, Basel, Switzerland) and analyses the image (confluence algorithm) to decide for further cultivation or for passaging. The results of a manual high- content screening experiment were compared with those obtained from the automatic system. The experimental variability was significantly smaller by using the automatic system. The Freedom Evo System, equipped also with the above-mentioned automated microscopic cell analyser, was used for isolation and culture of human primary (disc) cells, with similar yields, viability, and phenotype compared with the manual procedure[176]. By equipping individually the standard robotic cores with add-ons enabling them to process complex protocols, will give industry new applications and potential for innovation. But the reasonability of automating for smaller cultivation jobs was scrutinised in Hogan et al.[172] by defining a manual commitment time (MTC) for human intervention spent for machine versus real ''handmade'' cell cultures. Also, the possible overflow of cell-assay ready plates is discussed using the SelecT as an example.

Miniaturisation in biotechnology reached the market with the ambr system (Sartorius, Göttingen, Germany), a cell cultivation system which uses small volume bioreactors with integrated sensors for DO and pH, which provide individual closed loop control of these parameters. The automated pH regulation is possible through control of CO_2 and liquid alkali addition[178]. But miniaturisation requires first controlling how the assay can be adapted to the changes in the microenvironment of cells in the reduced volume[179]. Cell stress, for example, can be evaluated by the expression of stress markers[180].

From perfusion culture to the ''cell-nurse''

The knowledge of optimal growth conditions for microorganisms in fermentation can often be dated back to the beginning of civilisation. But even these cultivation routines were significantly improved since analytical methods for culture monitoring have become available, including molecular genetics. While for HTS and e.g. antibody production in CHO cells, well-known and stable cell lines are used, but more often cell culture characterisation is necessary, e.g. for the development of new applications or cell lines.

Batch fermentation uses all the volume of a bioreactor, which is not exchanged during the course of cultivation. While cells are growing, a deficiency in the energy substrates glucose and glutamine will be reached and the number of viable cells decreases, while the concentration of metabolic products like lactate and ammonia increases. The product of the fermentation is extracted after the end of cultivation[181].

Fed-batch cultivation offers a minimum of ''cell-nursing'' by feeding new medium to the bioreactor, because an initial low filling level allows this procedure for a limited time. Samples are drawn from the bioreactor to find the right time for the addition of new medium. Also most cell expansion protocols for HTS follow this kind of fermentation.

Perfusion culture means, that a medium is continuously exchanged while the cells are retained in the bioreactor by means of a membrane or fibres[182]. The advantage of the perfusion method is, that products can be harvested and metabolic waste products are removed simultaneously. Additionally, glucose, glutamine, and lactate concentrations can be measured in the perfusate, and feed additions and perfusion rate can be adjusted daily to keep the residual glucose and glutamine concentration in a favourable higher range. The Wave Bioreactor, a disposable polymer bag with a floating membrane, is moved by means of a platform, which also is used for heating. In the study of Adams et al.[183] and Sciences GEHL[184], the application of this perfusion bioreactor for protein production in CHO cells is described. In Hu et al.[185], all three bioreactor types were used for the same cell line and the cell cultivation qualities were compared. Cell viability is best and cultivation duration is longest in the perfusion bioreactor. The perfusate was analysed once a day on different analysers or kits. Thus, external daily concentration measurement and adjusting the perfusion rate once a day according to the lab results improves the culturing quality.

Apparently, continuous monitoring of glucose and lactate and especially of glutamine in this case, would have brought further advantages. Continuously analysing the perfusate enables for a fine-tuning of the chemical cell environment: Instead of adding medium – what means adding a fixed mixture of nutrition substances – the frequent addition of calculated amounts of concentrated stock solutions will maintain the nutrition situation of the cells at preset levels and will avoid ,on the other hand, the very high perfusion rates necessarily required at higher cell densities[186].

The technical feasibility of the on-line monitoring of these key micro-environmental parameters has been shown by Moser et al.[187]. In the work by Moser and Jobst[188], we reviewed the monitoring with (bio)sensors in disposable bioreactors and presented Jobst Technologies' contribution to this field: A miniaturised, disposable multi-parameter biosensor arrays for glucose, lactate, and glutamine/glutamate monitoring in a micro-flow system with few-microliter internal volume comprising micro-pumps. Various different physical formats of the devices, that are factory pre-calibrated, serving different application scenarios, can be fabricated. Also integration of the sensors during disposable bioreactor bag assembly is feasible because gamma irradiation together with the bag is possible.

Figure 3 shows how glucose levels can be controlled in a feedback loop using the glucose biosensor, Figure 4 how the full panel of analytes is controlled by the array of biosensors. An important prerequisite are the miniaturised pumps developed recently for low volume applications of this kind of applications.

Drug development

State-of-the-art small molecule targeting HIF/hypoxia signalling

Tumour hypoxia presents a major challenge to the cancer biologist from a therapeutic perspective. First, most solid tumours characteristically contain areas of hypoxia that are associated with metastatic disease and resistance to treatment[189]. Second, increased hypoxia occurs within the tumour microenvironment in response to treatment, providing a mechanism by which tumour cells can evade growth inhibition. There is a clear unmet need for therapeutic strategies that improve current treatment outcomes by exploiting the hypoxic response. Targeting the HIF pathway inhibits tumour progression *in vivo*[189] and can block unwanted effects of therapy-induced tumour hypoxia observed with γ-radiation[190,191] and other therapies used clinically (e.g. bevacizumab) resulting in significantly improved efficacy of these treatments in pre-clinical models[190,192].

Identifying small molecules through cell-based screening

There are several sites in the HIF pathway that are potential intervention points for inhibition by small molecules[189]. These include inhibition of HIF-1α stability or protein synthesis, or interference of HIF-dependent interactions[189,193–195]. A number

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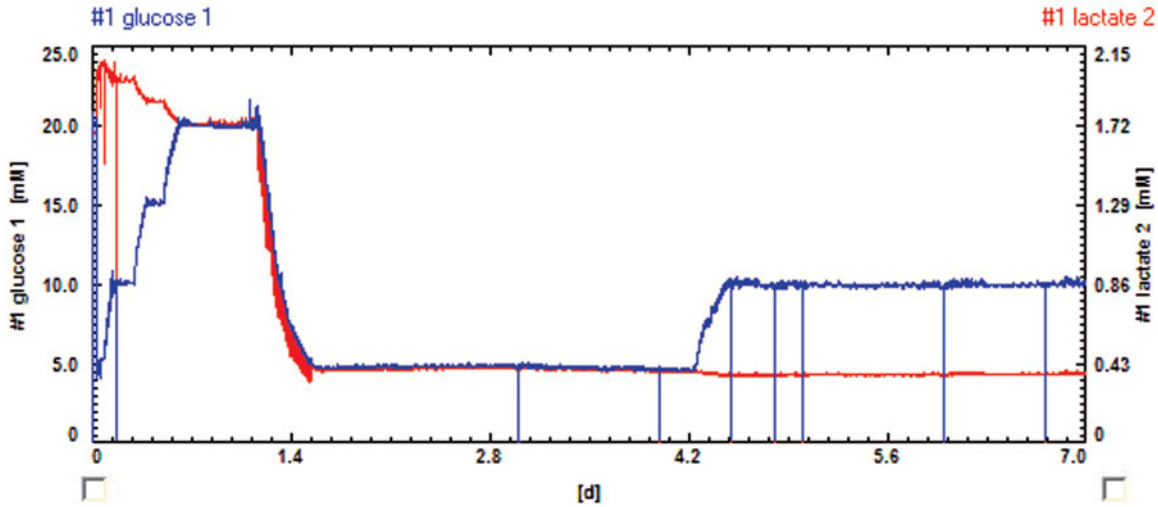

Figure 3. Screenshot of bioMON software client window – exposing glucose and lactate concentration on line traces to the user.

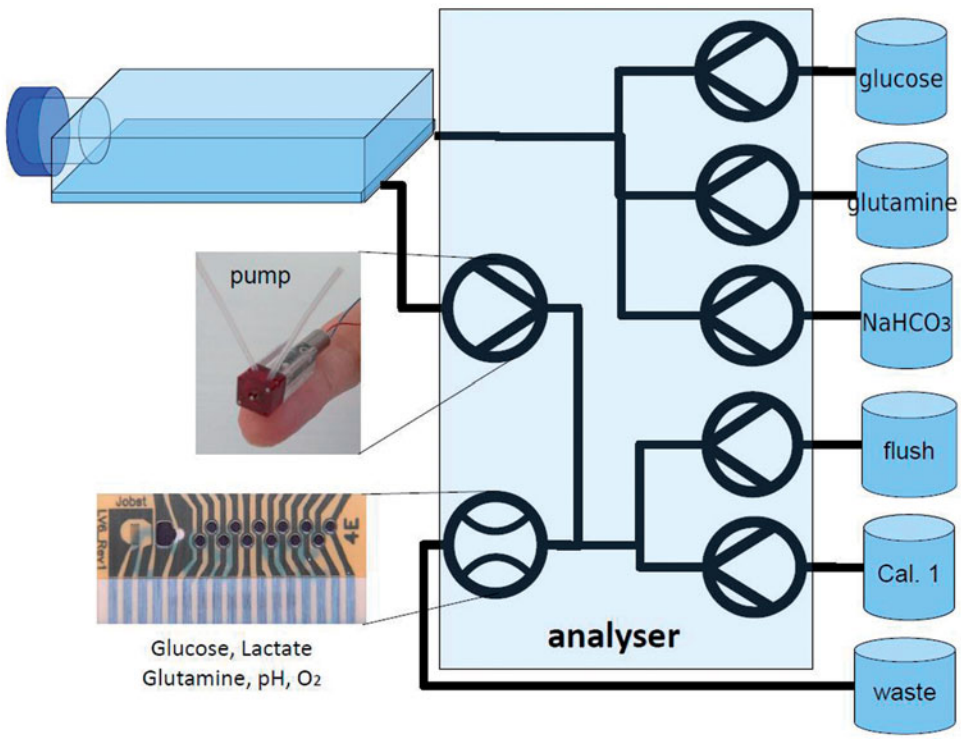

Figure 4. Indicating how the full panel of analytes is controlled by the array of biosensors.

of small-molecule inhibitors of HIF have been described, although their exact mechanism of action remains to be elucidated[189]. In addition, cell-based high-throughput screens are being used to identify novel small molecule inhibitors of HIF[189]. Generally, these systems utilise cells transfected with multiple HREs linked to a specific reporter gene construct. Cells express the reporter (e.g. luciferase or β-galactosidase) in a HIF- and hypoxia-dependent manner. This allows efficient screening of large libraries of compounds for HIF-inhibitory activity. Several small molecule agents identified from cell-based screens that indirectly inhibit HIF signalling have primarily been used to probe the HIF pathway rather than being explored as drug development candidates[189].

We were one of the first groups to publish the identification and characterisation of novel small molecule inhibitors of the HIF pathway using a cell-based screen that we developed[191]. Through this approach, we have successfully identified and mechanistically evaluated novel chemical series that exhibit desirable properties for therapeutic development (e.g. novel and chemically tractable, no attributable non-specific activity, no toxicity, good bioavailability, efficacious at inhibiting tumour growth consistent with blocking the HIF pathway *in vitro* and *in vivo*) and block the unwanted effects of treatment-induced tumour hypoxia[190,191]. We previously identified a novel HIF pathway inhibitor, NSC-134754[191] which we have used extensively as a tool compound for probing the HIF pathway[196,197]. Our chemical synthesis of NSC-134754 has led to the re-assignment of its chemical structure recently[198]. Further evaluation of the mechanistic, pharmacological and biological properties of NSC-134754 has provided invaluable insight for implementing a pre-clinical development pathway for the progression of other novel inhibitors that target HIF/hypoxia signalling as potential therapeutic agents.

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Targeting HIF and the p53/HDM2 pathway

The p53 tumour suppressor protein is induced and activated in response to a variety of cellular stressors. p53 is a potent negative regulator of HIF, and we have shown that pharmacological activation of p53 blocks HIF-mediated responses, tumour growth and angiogenesis *in vivo*, and induces significant tumour cell apoptosis in hypoxia[199]. Therefore understanding how cell death responses are regulated in tumour cells by HIF and p53 pathways is of particular interest to us[199–201]. Activity of wild-type p53 is usually lost due to deregulation of HDM2, an E3-ligase and well-known target of p53. HDM2 status is therefore an important consideration when determining how tumour cells may respond to therapy[202]. We have investigated the relationship between HDM2 and the HIF pathway[197,203], and the effects of HDM2 inhibitors on HIF have been described previously[204–206]. Nutlin-3 stabilises p53 by targeting the p53 binding pocket on the surface of HDM2 and shows potent *in vivo* anti-tumour activity in xenografts[204–206]. We have found that up-regulated HDM2 expression positively regulates HIF[203], HDM2 inhibitors also block angiogenesis through p53-dependent[207] and p53-independent mechanisms[208]. Nutlin-3, for example, shows greater efficacy in hypoxic cells with wild-type p53, only in combination with radiotherapy[204].

Most small molecule agents that have been designed to induce tumour cell death by activating p53 demonstrate poor activity in hypoxia and are often used in combination for therapeutic efficacy. Interestingly, however, we have found that the small molecule activator of p53, RITA (reactivation of p53 and induction of tumour cell apoptosis) can induce significant p53-dependent tumour cell death in normoxia and hypoxia as well as activating p53-dependent DNA damage responses[199,201]. Furthermore, DNA damage pathways that are induced by RITA also elicit cell cycle checkpoints involved in maintaining genomic integrity in response to stress[209].

Close links between HIF and p53 pathways have been shown in studies involving renal cell carcinoma[197,210]. Almost 80% of renal cell carcinomas occur due to the biallelic inactivation of the *von Hippel-Lindau* (*VHL*) tumour suppressor gene[211,212]. Renal cell carcinomas and hemangioblastomas usually express wild-type p53 as well as high basal levels of HIF-α due to loss of VHL function and are highly aggressive angiogenic, and metastatic cancers that remain resistant to radiotherapy and chemotherapy[211,212]. Although loss of VHL function leads to constitutive stabilisation of HIF-α, pVHL also has HIF-independent tumour suppressor functions involving other cell cycle and apoptosis pathways[213]. pVHL not only associates with HIF-1α to target it for proteosomal degradation[214], but has also been shown to directly associate with p53 and regulate p53 transcription in a HIF-independent manner[213]. By binding to p53, pVHL inhibits p53 degradation by HDM2[213]. It seems that loss of pVHL function has a critical role in promoting renal cell carcinoma by not only up-regulating the HIF pathway, but also by affecting the p53 cell cycle and apoptotic pathways. These pathways have a central role in promoting tumourigenesis in renal cell carcinoma and other hereditary cancer syndromes whereby pVHL is deregulated[211,212].

Harnessing the HIF/hypoxia response via novel mitochondrial mechanisms

Rapid advances in understanding metabolic switches in cancer cells has led to the development of inhibitors that sensitise tumour cells to cell death by disrupting the energy balance within mitochondria. Signalling molecules and numerous tractable targets that are critical for tumour cell metabolism are being explored for therapeutic intervention. For example, 2-deoxy-D-glucose (2-DG) targets the dependency of cancer cells for glucose and has been shown to sensitise tumours to radiation therapy and chemotherapy[215,216]. In addition, proteins involved in mitochondrial function are also being targeted[217]. Recently, using a functional genomics approach to identify novel regulators of HIF, we characterised the functions of the human CHCHD4 (coiled-coil helix coiled-coil helix (CHCH) domain 4) mitochondrial proteins, also known as MIA40[218]. Modulation of CHCHD4 protein expression was shown to regulate cellular oxygen consumption rate and metabolism[218]. Importantly, knockdown of CHCHD4 (MIA40) blocked HIF signalling in hypoxia and significantly inhibited tumour growth and angiogenesis *in vivo*[218]. Furthermore, in human cancers we found that increased CHCHD4 expression significantly correlated with the hypoxia gene signature reduced patient survival[218]. Further studies exploring the relationship between CHCHD4, mitochondrial function and the hypoxic response in tumours are underway, with a view to identifying novel therapeutic strategies to improve the treatment of hypoxic tumours.

CAIX-inhibitors

The development of metastasis is responsible for 90% of deaths from solid tumours[219], which has prompted the search for druggable targets with good anti-metastatic effects. In recent years, carbonic anhydrase (CA, EC 4.2.1.1) IX (CAIX) has been shown to be a potential candidate. In normal tissues, abundant CAIX expression is restricted to the glandular mucosa of the stomach where it is regulating the extracellular pH[220]. In most solid tumours hypoxia (i.e. partial or complete deprivation of oxygen in tissue) is by far the most important stimulator of CAIX expression[221]. Clinical biopsy material and clinic-pathological data across a large selection of cancer types including those of cervical, kidney, breast and head & neck cancer origin mostly support CAIX as a poor prognostic marker in patients with metastatic cancer. The role of CAIX in pH regulation, results in acidification of the tumour microenvironment which reduces cell adhesion, increases motility and migration, induces neo-vascularisation, activates proteases and enhances other hypoxia-induced processes[221]. While a role of CAIX in local control has been well established, it is less obvious from reports in the literature whether CAIX and acidosis also promotes metastatic dissemination.

Dual-action compounds including hypoxic radio-sensitisation

High levels of CAIX expression have been associated with poor prognosis, tumour progression and aggressiveness[222]. Since CAIX is implicated in both extra – and intracellular pH (pH_i) regulation, targeting CAIX through inhibition of its enzymatic activity using specific pharmacological inhibitors is a logical interesting approach[223]. Previously, it has been shown that these inhibitors require not only CAIX expression but also CAIX activation, the latter dependent on the tumour oxygenation status[34,224–226]. Several single-action compounds have shown inhibition of primary tumour growth and/or metastasis formation as single treatment[34,35,227–229] or in combination with conventional therapies[34,221].

Since CAIX activation is enhanced in low oxygen conditions, specific targeting towards and sensitising of these hypoxic tumour regions is an important prerequisite for new compounds. Recently, dual-action compounds with high affinity for CAIX based on the combination of a nitroimidazole and a CAIX targeting moiety have been designed[230,231]. Nitroimidazoles have been shown to improve the radiation response in terms of loco-regional tumour control and disease-free survival both when administered in a single or fractionated radiation schedule, with the

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5-nitroimidazole being less toxic compared with its 2-nitro analogue[232]. From a series of nitroimidazole-based sulphamides, a novel nanomolar dual-action compound (*N*-[2-(2-methyl-5-nitro-imidazol-1-yl)ethyl]sulphamide) was identified which showed the most pronounced *in vitro* reduction in hypoxia-induced extracellular acidosis[230]. Similar to single-action compounds, the dual-action compound was able to reduce tumour growth in a CAIX-dependent manner[34,230,231]. Due to the reduced extracellular acidification upon compound incubation, weak-basic chemotherapeutics have an increased potential to enter the cell, as exemplified by the sensitisation of tumours to doxorubicin[230]. Interestingly, the dual-action compound was able to enhance the therapeutic effect of irradiation with higher sensitisation enhancement ratios compared to the well-established hypoxic radiosensitisers misonidazole and nimorazole[231]. High bioavailability for oral formulations of the dual-action drug has been demonstrated, making potential clinical usability more patients convenient.

pH-regulation

Intracellular pH (pH_i) regulation is a fundamental process in living organisms and particularly in rapidly growing tumours which produce excessive amounts of H^+ via the lactic and carbonic acids metabolic endpoints. The hostile acidic and hypoxic tumour microenvironment is aggravated by a poor and chaotic vascularisation. These two inter-wined physiological processes, pH_i regulation and energy metabolism have high potential for the development of targeted cancer therapies. The reader will find a detailed approach of the complex network of pH_i regulation in recent comprehensive reviews[223,233,234]. In the context of this METOXIA project, we and others have designed experiments to validate, through genetic (shRNA, ZFN, CRISPR/Cas9-knockouts) and pharmacological approaches, the therapeutic potential of targets controlling pH_i.

Targets of interest: carbonic anhydrases CAIX, CAXII and monocarboxylate transporter complexes MCT1, MCT4, Basigin/CD147

Chiche et al. first validated the hypothesis that CAIX, when highly expressed, ensures a much more alkaline pH_i in cells[235]. This finding was independently confirmed in spheroids by the group of Adrian Harris[236]. We then discovered that in lung and colon carcinoma cell lines (A549, LS174), depletion of CAIX by shRNA leads to parallel up-regulation of the membrane-bound CAXII isoform which also plays a key role in pH_i regulation[235]. The mechanism behind the concomitant up-regulation of these two isoforms that in some cell types also concerns CAII is not yet resolved. Intracellular CO_2-sensing via soluble form of adenylyl cyclase is among the current hypotheses[237]. Consequently, the dual knockdown of CAIX and CAXII in some cells like the colon adenocarcinoma cell line LS174 led to the best reduction in tumour volume[235]. As reported in the previous section, we showed that combined knockdown of CAIX and XII radiosensitised tumour cells by increasing intracellular acidosis[238].

Lactic acid export, particularly in highly glycolytic and growing tumours, is another key determinant in the control of pH_i and tumour growth. Lactic acid is exported at a very low rate by simple diffusion of the uncharged acid form across the membrane and at high speed by H^+/Lactate$^-$ symporters called MonoCarboxylate Transporters (MCTs). Tumour cells are equipped with the ubiquitous MCT1 isoform and often with the hypoxia-inducible form MCT4. We and others[239,240] have shown that tumour cells that express only the MCT1 isoform (Ras-transformed fibroblasts, Myc-induced malignancies) could be severely inhibited by the specific MCT1/2 inhibitor AZD3965[241], which is now being utilised in clinical trials for small cell lung cancer[242]. In contrast, tumour cell lines (LS174, U87) expressing the MCT4 isoform that is well fitted for efficient lactic acid export[243,244] are fully resistant to AZD3965 or to expression of either shRNA-MCT1 or shRNA-MCT4[240]. Furthermore, we showed that ZFN-mediated MCT4 knockout sensitised LS174 cells to MCT1 inhibitor AZD3965. These findings demonstrated that simultaneous blockade of MCT1 and MCT4 that reduced pH_i by at least 1 unit (Marchiq I, Pouysségur J, in preparation) is mandatory to severely reduce growth of many human tumours[240]. This combined MCT1/4 targeting approach could as well be obtained by simple knockout of Basigin/CD147, an accessory protein required for appropriate folding and trafficking of MCT1/4 from ER to the plasma membrane[240,245].

MCTs inhibition: two anti-cancer strategies

AstraZeneca has now developed an MCT4 specific inhibitor. As was expected, when we combined these two MCTs inhibitors, we strongly reduced LS174 tumour growth phenocopying the double knockout MCT1/4 (Marchiq I, Pouysségur J, in preparation). Surprisingly these double LS174 MCT1/4-KO cells grow very slowly and do not die because they are capable of re-activating OXPHOS to maintain viable levels of ATP. This first strategy that blocks lactic acid export, glycolysis and tumour growth is likely expected to amplify anti-tumour action by the re-activation of T-cell immune response suppressed by acidic tumour environment[246].

The second anti-cancer strategy discussed recently[234], is to be used in a very narrow therapeutic window to limit cytotoxicity. It is based on the fact that disruption of both MCTs sensitised the cells to phenformin, a mitochondrial complex I inhibitor, inducing ATP crisis and rapid tumour cell death (Marchiq I, Pouysségur J, in preparation). These two strategies targeting pH_i regulation and bioenergetics will have to be validated in mouse genetics and immune-competent models before future clinical developments.

Chemical inhibitor synthesis

Some of the interesting CA-inhibitors which showed selectivity for the inhibition of the tumour-associated isoforms CAIX and -XII are compounds **1**–**9** shown in Figure 5. The tail present in a sulphonamide CA-inhibitor is essential for the binding of the inhibitor within the active site, and its influence on the inhibition profile against the many isoforms present in mammals is also significant. In fact, small structural changes in the ring on which the sulphonamide zinc-binding group (SBG) is appended, may also markedly influence the binding of the sulphonamide to the enzyme. This has been demonstrated in a recent work[247] in which the thienyl-carboxamido benzenesulphonamides **1** and **2** were crystallised bound to hCAII. Other interesting structure-based examples for obtaining isoform-selective CAIs of the sulphonamide type are compounds **3**–**9** which will be discussed here as tumour-associated, CAIX/XII-selective derivatives. Compound **3** incorporates tosylureido tails[248]. This class of derivatives has been reported earlier[248] but only recently an interesting selectivity ratio for inhibiting the transmembrane, tumour-associated isoforms (CAIX and -XII) over the cytosolic off-target one CAII has been observed[249]. Indeed, **3** has K_Is of 12 nM against hCAII, of 1.3 nM against hCAIX and 1.5 nM against hCAXII, whence, a selectivity ratio of 9.2 (hCA IX versus hCAII) and of 8.0 (hCA XII versus hCAII)[249]. However, the most CAIX/XII-selective compounds reported until now are those incorporating triazinyl tails, of which **4** is an interesting example[249–251]. Compound **4** has inhibition constants of 1098 nM against hCAI, of 37 nM against hCAII, of 0.75 nM against hCAIX and of 1.6 nM against hCAXII, whence selectivity ratios of 49.3 (CAIX versus CAII) and 23.1

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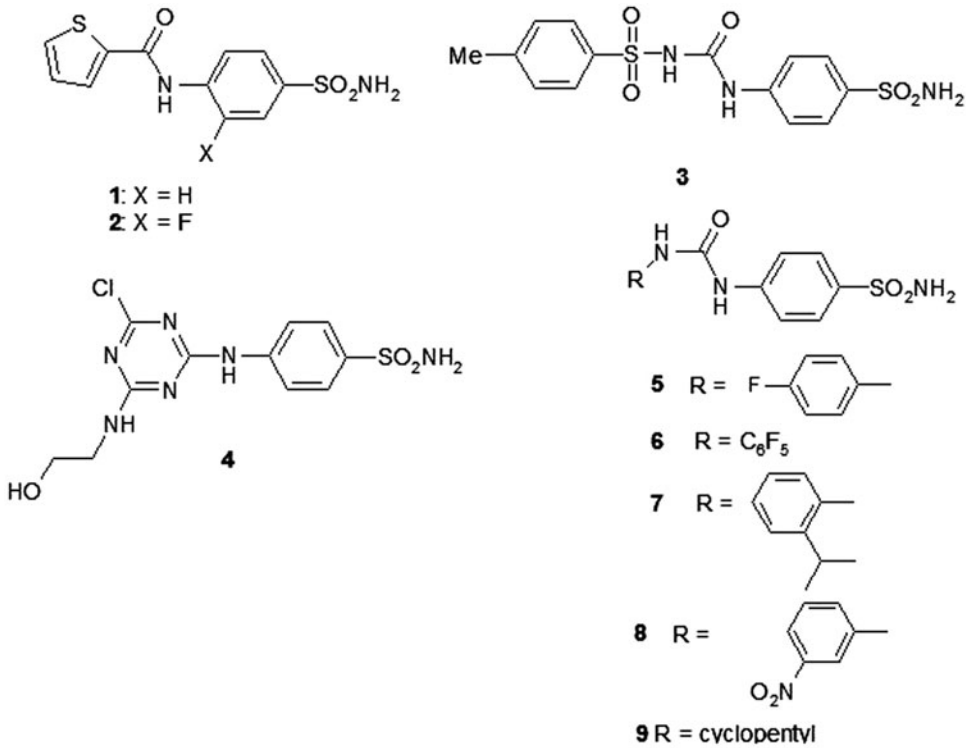

Figure 5. CA-inhibitors reported ultimately, which showed selectivity for the inhibition of the tumour-associated isoforms CAIX and –XII.

(CAXII versus CAII)[249]. Some congeners of **4** showed even higher such selectivity ratios (up to 700) for inhibiting the tumour-associated isoform hCAIX over hCAII[250,251]. But again it is interesting to compare this inhibition data with the X-ray crystallographic structure of compounds **3** and **4** in complex with hCAII[249]. The benzenesulphonamide fragment of the two inhibitors binds in the expected manner (coordinating to the Zn(II) ion) and was superposable for the two compounds. However, the tosylureido and substituted-triazinyl tails of the two compounds adopted extended conformations orientated towards opposing parts of the active site cavity[249]. This surely is reflected in the different inhibition/selectivity profiles of the two compounds, which have been mentioned above.

But undoubtedly, the most interesting case of CAIX-selective compounds is furnished by derivatives **5–9**, which incorporate again the benzenesulphonamide head, but this time 4-aryl/alkylureido tails[252,253]. A large series of such compounds has been reported[252] and for many of them good selectivity ratios (in the range of 16–53) for inhibiting CAIX over CAII were detected[252]. Another interesting feature for this series of compounds was that some of its members were also excellent hCAII inhibitors (in addition to strongly inhibiting the tumour-associated isoforms hCAIX and -XII, in the low nanomolar range)[252,253]. For example, the inhibition constants of compounds **5–9** against hCAII were of: 96 nM, 50 nM, 3.3 nM, 15 nM and 226 nM, respectively[253]. These parameters for the inhibition of hCAIX were of 45 nM, 5.4 nM, 0.5 nM, 0.9 nM and 7.3 nM, respectively. By reporting the X-ray crystal structures of the adducts of these five sulphonamides complexed to hCAII (Figure 6), it was observed that again the benzenesulphonamide fragments of the inhibitors are quite superposable, whereas the tails adopted a variety of conformations and orientations within the enzyme active site. This variability of binding is probably made possible by the flexible ureido-linker between the benzenesulphonamide part and second moiety of the 1,3-disubstituted ureas **4–9**. Indeed, some of the groups from the terminal part of these molecules were observed in the hydrophobic pocket, others in the hydrophilic one

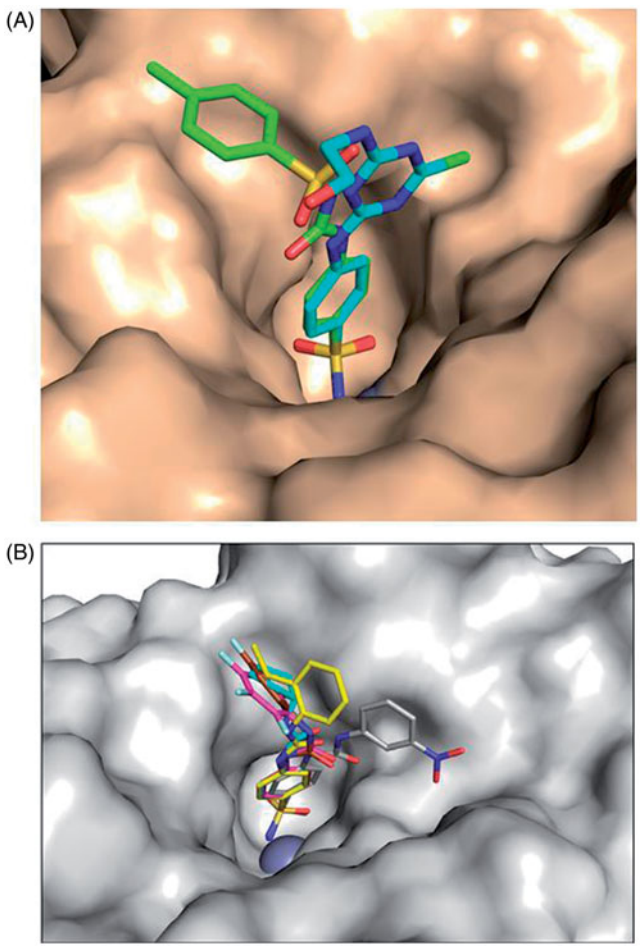

Figure 6. hCA II complexed with the tosylureido benzenesulphonamide **3** (green) and triazinyl-substituted benzenesulphonamide **4** (blue)[249] (A); and with five ureido sulphonamides **5–9**, compounds **5** (orange), **6** (pink), **7** (yellow), **8** (grey), and **9** (cyan) (B)[253].

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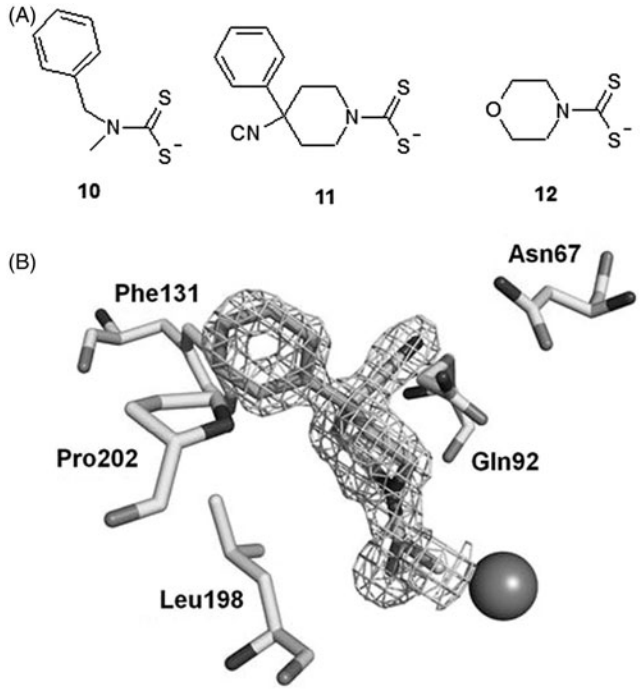

Figure 7. (A) Structure of DTCs **10–12**. (B) Electronic density for the adduct of dithiocarbamate **11** bound within the active site of hCA II [i]. The zinc ion is shown as the central sphere and the amino acid residues involved in the binding are evidenced and numbered (hCA I numbering system)[255].

and some of them between these two binding sites. Such different orientations may explain the range of inhibitory activity against hCAII (3.3–226 nM) and obviously hCAIX, as well as the selectivity ratios for inhibiting the two isoforms. However, a very interesting feature of some of these compounds (e.g. **8**) was their potent inhibition of growth of primary tumours and metastases in an animal model of breast cancer which potently over-expresses CAIX[252–254]. In similar tumour cell lines without CAIX, no inhibition of the tumour growth has been observed, which demonstrated that the drug target of these compounds is CAIX[252–254].

It may be observed from all data presented above that the sulphonamides dominated the drug-design landscape of CA-inhibitors for many years, However, very recently, new important chemotypes emerged. Among them, the dithiocarbamates (DTCs) are undoubtedly the most interesting ones[255–258]. These compounds have been rationally discovered as CAIs after our report of trithiocarbonate (CS_3^{2-}) as an interesting (milli – micromolar) CA-inhibitor. In the X-ray crystal structure of this inorganic anion bound to CAII, it has been observed a monodentate coordination of the inhibitor via one sulphur atom to the zinc ion from the enzyme active site, and a hydrogen bond in which another sulphur and the OH of Thr199 were involved. Thus, the CS_2^- was detected as a new ZBG. As DTCs incorporate this new ZBG, a rather large series of such compounds was prepared and evaluated for their inhibitory activity against several mammalian, fungal and bacterial CAs[255–258]. Several low nanomolar and sub-nanomolar CA-inhibitors were thus detected against all these isoforms. The X-ray crystal structures were also reported for three DTCs complexed to hCAII. DTCs **10–12** inhibited hCAII with K_Is of 25 nM, 41 nM and 0.95 nM, respectively, and hCAIX with K_Is of 53 nM, 757 nM and 6.2 nM, respectively[256]. As seen from Figure 7, the binding mode of the ZBG present in **11** is identical to that of trithiocarbonate (with one sulphur coordinated to the metal ion), but the organic scaffold present in the DTC was observed to make extensive contacts with many amino acid residues from the active site, which explains the wide range of inhibitory power of these derivatives (from the sub-nanomolar to the micromolar, for the entire series of around 30 DTC reported so far[255,256].

Preclinical models

Models for metastasis

Studying the anti-metastatic potential of anti-cancer compounds in appropriate models for metastasis as part of the preclinical drug development package is pivotal. *In vitro* cell spreading, migration and invasion assays are covering some of the aspects of the metastatic cascade and can be used to narrow down the search for the most potent lead compound(s). However, *in vitro* assays only recapitulate one or several aspects of the metastatic cascade and are by no means powerful enough to substitute for further *in vivo* testing of the lead compound(s). There are many different migration assays including the transwell (Boyden Chamber), scratch wound-healing, cell-exclusion zone, fence, micro-carrier-based and spheroid migration assays, which have been reviewed in great detail by Kramer et al.[259]. *In vivo* models for testing anti-cancer drugs can be grouped into intravenous, ectopic and orthotopic models. On some occasions genetically engineered (GM) mouse cancer cells have been generated in which over-expression or deletion of a particular gene enhances the metastatic potential. However, these GM cancer models often have a low incidence of distant metastatic disease and are unreliable for predicting clinical outcome of anti-metastatic therapy[260]. In intravenous models, a tail vein or intra-cardiac injection induces a systemic distribution of cancer cells. A commonly used variant of the intravenous model is the injection of colorectal carcinoma cells, e.g. HCT116 and HT-29 into the spleen. Splenic vessels which drain the spleen directly transport the cancer cells to the liver where well developed metastases are visible on magnetic resonance (MR) scans after 28 d[261]. These ''experimental metastasis'' model recapitulates some of the late steps of the metastasis cascade but specifically not the earlier ones including the selection pressure in the primary tumour, local invasion of the adjacent host tissue and intravasation of blood vessels. In particular the tail vein model gives a 100% incidence of lung metastases in a temperate reproducible manner and is therefore widely used for testing anti-cancer compounds. In contrast, there are the ''spontaneous metastasis'' models in which the tumour cells are either growing in a donor organ (=ectopical, usually sub-cutaneous) or in the organ of origin (=orthotopic) allowing to study the effects of micro-environmental factors on metastatic disease. The orthotopic model provides a more clinically relevant setting than the conventional sub-cutaneous model. As an example, MDA-MB-231 injected into the mammary fat pad induces spontaneous metastases in CBA nude mice[229,262].

The use of carbonic anhydrase inhibitors (CA-inhibitors) in enhancing local control and overall survival has been investigated for more than a decade. However until recently only broad-spectrum CAIs like acetazolamide (AZM or AZA) were available. *In vitro* research showed that AZM was very effective in reducing the invasiveness of renal carcinoma cells[263]. In addition, we showed in cell lines that highly expressed CAIX under anoxic conditions (e.g. the colorectal model HT-29 and breast cancer cells GM to over express CAIX [MDA435 CA9/18]) that AZM treatment increased the intra-tumoural pH. pH changes were accompanied by an enhanced uptake of weak-base doxorubicin which caused an increase in the doxorubicin-induced cytotoxicity[264]. However, AZM is not very specific for the tumour-

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associated CAIX/XII isoforms, inhibiting also the more generally expressed off-target CAI/II isoforms. This prompted the development of more selective CAIX/XII inhibitors. In the last few years, selective CAIX inhibitors based on the sulphonamide, coumarin and sulphamate classes have shown to have promising anti-metastatic potential. Preclinical studies with orthotopic 4T1 (breast carcinoma) tumours showed that treated with ureidosulphonamide 25 and 104 and coumarin glycosyl coumarins 204 and 205 targeted 4T1 metastasis and improved metastasis-free survival[35]. Belonging to the sulphamate class of CA-IX inhibitors, 4-(3′-(3″,5″-dimethylphenyl)ureido)phenyl sulphamate (also named S4) reduced the spreading and migration of human MDA-MB-231 (breast carcinoma) cells and significantly reduced the metastatic burden in lung of mice with well-established orthotopic MDA-MB-231 tumours[229]. The significant anti-proliferative effect of similar sulphamate -based CAIX inhibitors on a range of human breast cancer cells offers the potential for additional anti-cancer leads[265]. Besides CAIX other hypoxia-related targets like hypoxia-inducible factor 1 alpha (Hif-1α), glucose transporter 1 (GLUT-1) and vascular endothelial growth factor (VEGF) are good approaches for anti-metastatic effects[266,267].

Metastasis is a complex process consisting of multiple steps of malignant cell–host cell interactions, which are tightly regulated by various signalling pathways. The metastatic cascade includes interactions between invasive tumour cells and various host cells in the tumour microenvironment, intravasation of tumour cells into the circulation, transport of tumour cells to distal tissues and organs, extravasation of tumour cells from blood or lymphatic vessels, formation of initial metastatic niches, and re-growth of metastatic tumours. Despite the advances of image analysis to detect metastatic lesions at stages, clinically detectable metastases by imaging analysis often represent the end-stage of the metastatic cascade. Visualisation of the early events of cancer metastasis is almost impossible in mammals while zebrafish provide an alternative tool to study the early events of metastasis. Given the advantages of transparent features, immunoprivilege, and genetically manipulatable zebrafish embryos, a zebrafish metastasis model has been established[268]. In this model, human or mouse tumour cells can be color-coded with a fluorescent dye and their movement in the fish body can be detected at the single-cell level. Moreover, exposure of zebrafish in a hypoxic environment enhances cancer cell dissemination in this preclinical model[269]. Hypoxia-triggered VEGF-dependent angiogenesis is crucial for cancer cell dissemination. This model offers a unique opportunity to study the early events such as intravasation of tumour cells into the circulation and formation of metastatic niches.

Another important route of cancer spread is lymphatic metastasis by which tumour cells metastasize to regional lymph nodes. In many epithelial cell-originated tumours such as breast cancer, colorectal cancer, prostate cancer, ovarian cancer and lung cancer, lymphatic metastasis occurs more frequently than blood-stream metastasis. Despite this knowledge, appropriate animal models have been lacking to study the events and mechanisms that underpin lymphatic metastasis. In our laboratory, we have developed several tumour models in which lymphangiogenic factors are highly expressed[270,271]. Implantation of these tumour cells in mice results in lymphatic metastasis in regional lymph nodes. Consistent with lymphatic metastasis, tumour tissues contain high numbers of lymphatic vessels. It is likely that both intra-tumoural and peritumoural lymphangiogenesis contribute to lymphatic metastasis. To quantitatively assess the tumour-derived lymphangiogenic factors in promoting lymphangiogenesis, we further developed a mouse corneal lymphangiogenesis model[272]. This is a unique model to quantitatively study lymphangiogenesis induced by different factors either alone or in combinations, simply because cornea is an avascular tissue.

Preclinical cancer models for the assessment of novel therapeutics targeting hypoxia

The increase in knowledge of cancer pathogenesis has led to a growth in the development of anti-cancer drugs, but the successful pre-clinical assessment of novel cancer therapeutics is dependent on the use of relevant experimental models. Assessing any new drug requires methods that combine prognostic accuracy and high throughput/cost. Therefore, the models used differ at each stage of the process. It is estimated that one in 5000–10 000 potential anti-cancer agents is approved by the FDA and only 5% of those that enter Phase I clinical trials[273]. Even then many fail in Phase II and Phase III trials[274]. Therefore, there is an urgent need for superior, more clinically relevant human cancer models for drug assessment.

2D monolayer cell culture

The most practical starting point in the investigation of novel oncology therapeutics is the use of panels of cell lines that encompass the cancer of interest grown in 2D cell culture. This initial strategy allows the evaluation of drug on functional parameters such as cell proliferation, cell death and effects on invasive potential to be measured, using standard protocols and IC_{50} values to be obtained. This is exemplified by studies identifying CAIX inhibitors active against breast and ovarian cancer cell lines[265,275]. Possible additive and synergistic interactions with standard treatments can also be studied. Because cancer is an extremely heterogeneous condition, the size of the cell panel used in preliminary work can be crucial[276] and should include cell lines that reflect various sub-types of the cancer. The major drawback of many cancer cell lines is that a highly passaged line may no longer fully represent the initial primary cancer they were derived from, since selection pressures tend to allow less differentiated cells to survive, leading to loss of some biological differences[277]. These problems can be overcome by the development of cell lines *de novo*, from cancer tissues[278].

2D cell culture of cell lines is an extremely simple model system, and bears little resemblance to any *in vivo* physiological environment, and it cannot recapitulate the heterogeneity between or within human tumours. Single-cell sequencing shows that tumours contain individual clones with many different mutations[279]. Culture media maintain neutral pH, and therefore do not reflect the increased acidity of the tumour microenvironment which can drop to pH 6.0[280]. Further, most cells are cultured at oxygen levels of approximately 21%, when oxygen tension in normal tissue is 7% on average, and much lower in tumours, with the mean value of 1.5%[281]. Moreover, cells in 2D culture show considerable variation in morphology, metabolism, gene expression and signalling in comparison with cells in tumour tissue[282–285].

In solid tumours, cells adapt to survive in conditions where pH and O_2 gradients are the norm, glucose and other nutrients are limited, and waste metabolites concentrated. Therefore, cellular responses to drugs can differ markedly when cells are cultured using *in vitro* 3D methods rather than 2D culture[286,287]. However, the advantages of using 2D culture at early stages outweigh any disadvantages in that these models offer a fast, reliable and reproducible test bed and any results can be quickly investigated for specificity and potential mechanisms of action.

3D culture

While differences between 2- and 3D models are important in any anti-cancer drug study, they are crucial when the main strategy under investigation is the exploitation of survival strategies in hostile micro-environmental conditions, such as interference with pH homeostasis by CAIX-inhibition. Much current research

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considers the interaction of tumour cells with the microenvironment and how tumour-driven changes in the microenvironment then influence the activities of tumour cells. Therefore, relevant 3D tumour culture techniques are the next logical research step. These methods can be divided into *in vitro* or *in vivo* systems.

The development of 3D *in vitro* models, using spheroids grown from cell lines, or in matrix scaffolds, allows a better understanding of drug responses and intracellular signalling in a more relevant milieu that mirrors some aspects of tumour physiology and show better correlation with results from *in vivo* drug studies[282,287–291]. The methodology for 3D growth models is well documented using suspension approaches or ECM component systems, which allow a more physiological model of invasion[292,293]. The matrix can be designed to include components that mirror the stroma of the cancer under study, for example, breast cancer extracellular matrix has a high proportion of collagen I which has been shown to promote invasion and metastasis[294,295]. As spheroids become larger, areas of hypoxia and acidosis develop reproducing oxygen and pH gradients similar to those found in micro-metastases and avascular tumours, and causing the development of a necrotic core and perinecrotic hypoxic area[296,297]. The role for CAIX in regulating extracellular and pH_i in several cancer types has been demonstrated by use of 3D spheroids[298,299].

Xenograft and genetically engineered mouse models

Xenograft models allow tumour growth, drug efficacy, toxicity and a range of pharmacokinetic measurements to be made in an *in vivo* situation. Xenografts are however formed from the same cell lines used in 2D studies, and grown in immune-deficient mice to allow rapid tumour growth. While these growths can recapitulate the oxygen and pH gradients of tumours, the stromal elements are murine and therefore may not accurately reproduce the stromal–tumour interactions found in human tumours and xenografts from a cell line will not encompass the complete genetic and epigenetic heterogeneity of an actual tumour[300]. However xenografts allow the investigation of therapy using known histological cancer sub-types[301], but metastatic models are limited and duplication of the different tumour sub-types found in patients can be difficult, as this is dependent on the cell lines available[302,303]. However, reduction of breast cancer metastases to the lung by CAIX inhibition has been demonstrated using both the human MDA-MB-231 xenograft model[229] and the murine 4T1 mammary model[35].

Combination strategies can also be assessed pre-clinically such as the interaction between CAIX inhibition and either radiotherapy[231,238] or angiogenesis inhibitors[299]. The translation of xenograft studies into the clinic has had variable success, although better selection and characterisation of the model has led to better success in more selective targeted therapies[304,305]. Mechanistic insights can also be derived using xenografts, for example, to study the consequences of silencing CAIX or CAXII and demonstrating modified tumour growth[235].

Murine tumours can be induced in immune-competent mice; some occur spontaneously, or can be induced using carcinogens. The other strategy is to use GM mouse models (GEMMs) in which specific mutations are introduced which can be tissue specific, or temporally controlled[306]. GEMMs, allow the growth of primary cancers in immune competent mice within a murine tumour microenvironment[307]; this may more accurately replicate the stroma component and microenvironment of human cancer than xenografts, but they are not of human origin[308–310]. These models have been demonstrated to replicate closely the results of clinical trials for human cancers[311,312]. This system still has limitations and no animal model is feasible for HTS[287,313]. However, models such as the TRACK renal cancer mouse model which express a constitutively active HIF1α in kidney proximal tubule cells should provide enhanced insight into the oncogenic functions of HIF1α in renal cancer[314].

Ex vivo *tumour tissue explants/grafts*

One way of modelling cancer cells *in situ* is the use of *ex vivo* tumour material, known as tumour graft models or patient-derived xenografts[315]. These xenografts are formed by digesting the patient's tumour tissue and then transferring cells from it into an immune-deficient mouse. Tumours are then directly passaged from mouse to mouse.

The advantages of this system have been shown using tissue grafts from breast cancer patients, which have been shown to reproduce tumour growth, metastatic capacities and pathology of the main sub-types of human breast cancer in an accurate manner[316]. Studies indicate that they also preserve histological indicators such as receptor positivity and proliferation markers, suggesting that this methodology can sustain the original pathophysiology of the tumour[303]. This could be a relevant model for anti-cancer therapeutics that exploits micro-environmental changes. The major disadvantage of the system is that more than one animal is needed to capture the variability of each tumour and therefore multiple transfers are needed even for one tumour[317]; and while the tumour cells preserve many of the feature of the original tumours, there are alterations and losses of the human stromal elements of the tumour[318–320]. Other disadvantages are the substantial cost, since these grafts must be sustained in a murine host, and therefore passaging techniques require more skill, and also there can be a long growth period for a tumour graft to form[321].

Cultured ex vivo *tumour explants*

Primary tumour material can also be cultured *ex vivo* in a similar manner to 3D tumour spheroids[322,323]. This use of primary tumour tissue allows analysis of heterogeneous material and tumour sub-types. With this approach, there is no digestion of tissue so that these explants maintain the stromal structures associated with the original tumour[324]. This is a particularly useful system if tissue can be obtained pre-treatment from biopsy material.

Our own studies indicated that changes to breast cancer explants can be seen and monitored within 5 d in most instances. This system could define cases that will respond to a specific therapy and be used as part of the route towards personalised medicine. It may also provide insights into tumour response comparable to neoadjuvant trials[324]; since investigation of co-treatment strategies or scheduling of treatments may be examined. We have found that these explants can be maintained in culture for at least a month[323].

More research is currently needed into metastatic disease. *In vivo* metastatic research utilises injection of tumour cells and monitoring distal sites for metastatic formation[325]. These are time consuming assays that are expensive and do not allow observation of the early phases of metastasis[325,326]. *Ex vivo* explant methodology also allows monitoring of invasive changes in an appropriate tumour microenvironment which could be assessed in high throughput formats. Changes in matrix composition and the effect of stromal cells can also be assessed. The advantages of this system are that explant growth/response can be continuously monitored and tissue can be harvested and investigated when changes are actually taking place. Tissue can be lysed, or fixed for later analysis. The disadvantages are that pharmacokinetic changes cannot be monitored and analysis is time consuming. However, the use of patient-derived human tumour tissue for appropriate preclinical research is increasing[327].

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Summary – Section ''Preclinical cancer models for the assessment of novel therapeutics targeting hypoxia''

Although there have been many recent advances in cancer treatments, the number of novel therapeutics that fail during randomised phase III clinical trials of novel oncology therapeutic, even after successful completion of Phase I and II is extremely high[300,321,328]. This suggests that pre-clinical models of this disease either do not accurately reproduce authentic tumour physiology or fail to reflect the actual heterogeneity of tumours. One reason for this is a failure to explore the effects of new drugs in the conditions found in the tumour microenvironment, which strongly influences the growth and survival of tumour cells. The advantages and disadvantages of each model system suggest that novel therapies must be tested using several appropriate methods.

In the aspect of hosts, genetically identical mice are commonly used in both treated and non-treated groups. In humans, however, the genetic information in each individual patient is different from others. Additionally, the same age and gender mice are used for experimentation and human patients often are different in these parameters as well. For tumours, development of a clinically detectable tumour in human patients often takes years while growing a mouse tumour of proportionally the same size often needs a few weeks. The different growth rates could affect the cellular and molecular composition in the tumour microenvironment and eventually lead to different responses to the same drug. Also, in experimental tumour models established tumour cell lines are often used and they may lose their identity of the tissues and organs from which they originated. Another concern for the mouse tumour model is that tumours are often implanted subcutaneously and this location is not directly relevant to human patients. As for treatment regimen, treatment with drugs is often initiated shortly after tumour implantation in mice and the same drugs are often given to human cancer patients with advanced and metastatic cancer disease. Finally, assessment of therapeutic efficacy in human cancer patients and mouse tumour models are different. In human patients, survival especially overall survival improvement is the endpoint for drug approval. In mice, tumour size has been used as a surrogate marker for therapeutic efficacy. Tumour sizes do not always correlate with survival advantages. Within the context of radiotherapy it is often more relevant to use TCD50 experiments, although these are very expensive. Although various genetic mouse models have been established to reproduce the clinical situation, these genetically manipulated tumour models are often driven by a particular oncogene or mutations of tumour suppressors and are thus less relevant to the clinical situation.

A novel strategy has been proposed called ''co-clinical trials''[329], which synchronises preclinical and clinical trials by correlating human patients and mouse models in parallel. A model using *ex-vivo* patient derived explant models either in a murine host or in *ex-vivo* culture could realistically offer a more pre-clinically appropriate testing bed for clinical trials or in concert with co-clinical trials[276]. Furthermore for drugs targeting tumour vasculatures, it is important that orthotopic cancer models should be considered since blood vessels in various tissues are intrinsically different from each other. An alternative is also spontaneous models arising from transgenic animals.

Clinical testing

Diagnostics: hypoxia detection and imaging

Radiotherapy combined with chemotherapy is currently the preferred treatment modality for patients with solid tumours. Treatment effectiveness however is dependent on tumour microenvironmental characteristics, such as hypoxia. Hypoxia results in poor prognosis since it promotes resistance to radiotherapy and chemotherapy and it increases tumour aggressiveness, angiogenesis and metastatic potential. Detection and quantification of hypoxia could therefore help selecting patients who might benefit from treatment adaptation counteracting hypoxia.

Since tumour hypoxia is distributed very heterogeneously, biopsy-based assessment of hypoxia may be prone to sampling errors. Furthermore, for some treatments, like selective dose escalation to hypoxic tumour sub-volumes, gene expression is clearly not appropriate to guide treatment. Therefore, PET-based assessment of tumour oxygenation in hypoxic tissue which allows assessment of the whole tumour and 3D mapping of the distribution of hypoxia, is attractive and has received immense interest.

In the context of radiotherapy treatment planning, PET imaging of the selective binding and retention of 2-nitroimidazoles prior to and during treatment is well-suited for displaying the spatial distribution of hypoxia[330]. As an increased radiation dose to radio-resistant hypoxic areas may increase local control[331], accurate identification and stable detection of intra-tumoural hypoxic sub-regions is utmost important[332].

Some inherent weaknesses in PET in general (low resolution of several mm) and hypoxia PET in particular (slow tracer retention and slow clearance of unbound hypoxia-unrelated tracer) leads to limited inter-tissue and intra-tumoural contrast even hours after tracer administration, which may compromise the quantitative accuracy of hypoxia PET. Specifically problematic is the risk of overlooking areas where viable hypoxic cells are intermixed with necrosis with little tracer at a spatial scale that is too small to resolve on PET. Interestingly, although PET hypoxia imaging is able to identify patients with poor prognosis, the 15-gene hypoxia gene signature described below ranks patients differently than PET imaging, suggesting that they do not provide identical information[333]. In the METOXIA project, significant efforts to develop hypoxia tracers with improved pharmacokinetics has been undertaken, resulting in the development and testing of two new tracers. Pimonidazole, a 2-nitroimidazole, has excellent pharmacokinetic characteristics and is often used as the ''gold standard'' to mark hypoxia in tissue following tissue removal, sectioning and immunohistological labelling. However, since radioactive labelling of pimonidazole has not been attempted previously, a method for labelling of pimonidazole was developed under the framework of the METOXIA project[334]. Initial *in vitro* testing in cell lines revealed that [^{18}F] labelled pimonidazole ([^{18}F]FPIMO) was strongly hypoxia-driven with slight superiority to the established hypoxia tracer [^{18}F]FAZA. [^{18}F]FPIMO also accumulated in experimental tumours compared to non-hypoxic reference tissue and its intra-tumoural distribution (autoradiography) was similar to the distribution of unlabelled pimonidazole (immunohistochemistry). Nonetheless, [^{18}F]FPIMO proved inferior to [^{18}F]FAZA *in vivo*, since absolute tumour signal and intra-tumoural contrast was low, thus compromising the quantitative accuracy of PET imaging. Low tumour signal was coupled to extensive tracer accumulation in liver and kidneys, and very rapid degradation of [^{18}F]FPIMO in the blood. On-going work focuses on the possibility of labelling pimonidazole in different positions with [^{18}F] to improve tracer stability *in vivo*.

A recently developed 2-nitroimidazole nucleoside analogue [^{18}F]-HX4 with preferred pharmacokinetic properties, having high water solubility and fast clearance from non-hypoxic tissues, has shown to be a promising and non-toxic marker to visualise tumour hypoxia[335–337]. Preclinical studies in a rat rhabdomyosarcoma model demonstrated increasing [^{18}F]-HX4 contrast over time, reaching a plateau 4 h after injection[335]. A recent clinical trial in NSCLC patients confirmed this optimal imaging time point. Tumour hypoxia, defined as a tumour-to-blood ratio

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(TBR) > 1.4, was observed in 80% of the primary tumours and 60% of the involved lymph node regions increased significantly from 2 h to 4 h after injection both for the primary tumour and the lymph node regions[338]. Additionally, it was found that the minimal acquisition time without affecting TBR and hypoxic fraction equals 10 min, reducing influence on image acquisition due to patient movement[338].

Recently, it has been pointed out that a systemic examination of several 2-nitroimidazole based PET hypoxia markers within the same animal model or patient group is needed to assess whether one tracer is superior to another[339]. Several of these studies are currently on-going and focus on optimal imaging time point, TBR, spatial reproducibility and oxygen sensitivity as endpoints. Especially for future patient dose painting studies it is important to acquire insights in the spatio-temporal stability of the PET marker, which would imply image acquisition in treatment position in order to reduce possible registration errors.

Assessment of tumour hypoxia using hypoxic metagenes

Generally the use of gene expression profiling is a powerful diagnostic approach to identify and quantify tumour hypoxia and provides a number of distinct advantages over the other techniques described. Gene expression profiling is done by extracting RNA from clinical samples and simultaneously determining gene expression of multiple transcripts. Traditionally, this was achieved by using a gene expression microarray platform. Although the very large datasets generated using this approach have been necessary for deriving and validating hypoxia signatures, microarrays provide too much data and are not a GCP validated format for routine diagnostic use. Therefore, recent efforts to apply and validate hypoxia-associated gene signatures (or metagenes) have utilised low density quantitative real-time polymerase chain reaction (qPCR)-based assays to interrogate the expression level of fewer genes.

The main strength of this approach is the ability to measure multiple hypoxia responsive markers in parallel, providing a more robust measure of hypoxia than determining the expression of a single gene that is hypoxia-inducible, e.g. CA9. This property means that metagene analysis produces hypoxia measurements with lower levels of intra-tumour heterogeneity[340].

Using Affymetrix U133plus2 GeneChips expression data from 59 head and neck squamous cell cancers (HNSCC) our Oxford partner group initially developed a 99-gene hypoxia signature[341]. The signature was derived using a seed clustering approach, where a hypoxia gene sub-network is formed by selecting gene transcripts whose expression is highly correlated with the expression of 10 well-known hypoxia-regulated genes (the initial ''seeds'' of the network). High hypoxia score (HS), i.e. high summary expression of the hypoxia signature, was able to predict worse outcome in an independent cohort of 60 HNSCC cases. Further validation using a published series of 295 breast cancer samples also confirmed the ability of the hypoxia metagene to significantly predict disease-specific survival.

A more compact 51-gene hypoxia signature was developed extending the above approach to a meta-analysis context where genes were selected in a common hypoxia signature if they were highly correlated with the expression of the initial seeds and this correlation was consistent across cancer datasets and types[342]. More than 1000 cancer samples were used for this analysis and the resultant metagene was able to better predict outcome in several large independent data sets than the initial 99-gene signature and other published signatures. Specificity of the signature for hypoxia has also been demonstrated using cell lines exposed to hypoxia.

Recently, we have applied this signature to the Metabric cohort of 2000 breast cancers (Figure 8)[343]. This work demonstrated that basal-like and HER2 + breast cancers have increased hypoxia scores compared with luminal A, luminal B, normal-like breast cancer sub-types and normal breast tissue. Consistently, the basal-like sub-type of breast cancers has been reported to have high HIF-1α activity[344]. In this dataset the hypoxia signature was highly prognostic.

The prognostic information provided by hypoxia signatures could allow clinicians and patients to make better informed decisions when planning treatment strategies and this information may be particularly useful in cases where routine histological analysis is unable to provide prognostic value[345]. However, a more powerful application for hypoxia signatures is their use to predict which patients will respond to hypoxia-modifying or hypoxia-targeted treatments.

A 26-gene hypoxia signature was able to retrospectively identify laryngeal cancer patients that benefited from hypoxia modifying carbogen and nicotinamide (CON) treatment in combination with radiotherapy[346]. Patients with low hypoxia scores did not benefit from addition of CON to the standard accelerated radiotherapy treatment. When the same analysis was conducted using samples from a bladder cancer trial there was no benefit of addition of CON treatment in patients with hypoxic tumours. There are several potential reasons for this lack of benefit. One possibility is that while the hypoxia signature used may accurately detect hypoxic cells in laryngeal cancer, it may not be accurately detecting ''hypoxic'' cells in bladder cancer, i.e. bladder cells may respond to hypoxia by inducing a different set of genes. Another possibility is that the actual radio-resistant fraction of cells in the tumour is not proportional to the hypoxia score.

The amount of hypoxia in tumours is expected to decrease in response to anti-proliferative agents as oxygen demand is reduced. Indeed, a reduction in the hypoxia metagene score was observed in breast cancers when patients were treated with an aromatase inhibitor and this change correlated significantly with a reduction in a marker of cellular proliferation (Ki67)[347]. This increase in tumour oxygenation may improve the efficacy of subsequently administered treatments, e.g. radiotherapy or may reduce the efficacy of agents that require hypoxia for their activation, e.g. hypoxia-activated prodrugs.

An alternative marker for assessment of hypoxia is microRNA-210 (mir210). Expression of mir210 is strongly induced in cells exposed to hypoxia in a HIF-dependent manner[348,349]. mir210 has been demonstrated to target several transcripts including the mitochondrial iron sulphur scaffold protein ISCU[350,351]. The down-regulation of ISCU by mir210 during hypoxia was shown to repress mitochondrial respiration, providing an adaptive response to the hypoxic stress.

Levels of mir210 expression in 219 breast cancer cases correlated strongly with the 99-gene hypoxia metagene suggesting it is a useful alternative for measuring tumour hypoxia[348]. Consequently, high levels of mir210 correlated with poor prognosis in breast cancer[352]. This finding was confirmed in an independent study of breast cancer cases, with mir210 providing prognostic performance equivalent to that of many multiple genes signatures[353]. In contrast, high levels of mir-210 expression correlated with better prognosis in renal cell carcinoma reflecting differences in the tumour biology of breast and renal cancers[349].

In cervical cancer, a combined analysis of DCE MRI and gene expression profiles was performed to generate a 31 hypoxia gene signature with prognostic impact in a cohort of patients referred to chemo-radiotherapy[354]. Its specificity for hypoxia was validated in analysis with cervical cancer-specific hypoxia gene sets derived from cell lines. The signature predicted outcome in an

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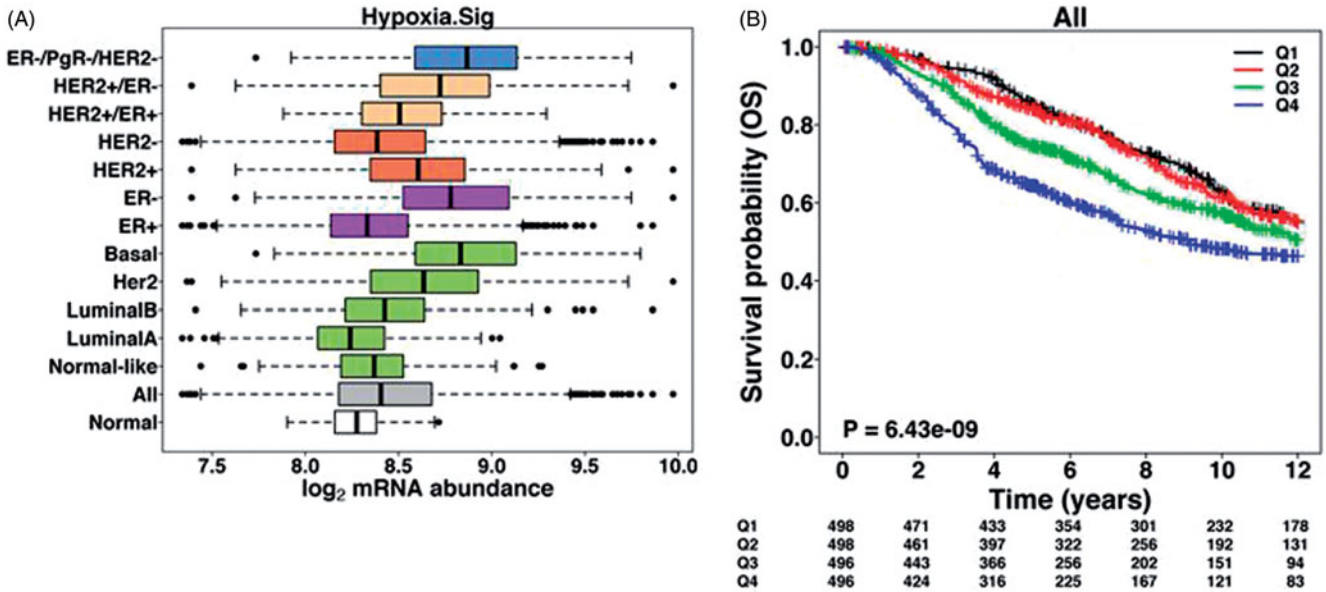

Figure 8. Validation of the common hypoxia signature[342] in different breast cancer subtypes and prognostic utility. (A) Box-whisker plots showing median, upper and lower quartiles and 95% range of hypoxia signature. Breast cancer subtypes were classified by hormonal receptor status (ER−/PgR−/HER2−, HER2−, HER2+, ER−, ER+) or PAM50 (Basal, Her2, LuminalB, LuminalA, Normal-like). (B) Kaplan–Meier analysis of the Metabric cohort demonstrating poorer overall survival for breast cancer patients with relatively higher hypoxia signature score. Patients were divided into quartiles from low (Q1) to high (Q4) hypoxia groups based on cumulative hypoxia score.

independent cohort and showed significance in multi-variate analysis with conventional clinical markers. Its relationship to a prognostic DCE MRI parameter suggests a potential of combining MRI with the hypoxia gene expression signature in treatment planning of this disease.

In prostate cancer, the possibility of generating a gene expression signature of HIF1 targets for monitoring changes in hypoxia during androgen-deprivation therapy (ADT) was investigated. ADT improves outcome of intermediate and high-risk patients when combined with radiotherapy in a neoadjuvant setting, and this has been attributed to increased oxygenation during the neoadjuvant period[355]. Still, a significant proportion of patients relapses, and a hypoxia biomarker could be useful for planning radiotherapy initiation and a possible need for concurrent hypoxia targeted therapy. In a xenograft model system, we demonstrated ADT-driven down-regulation of HIF1 target genes without changes in the hypoxic fraction[356]. The down-regulation was probably a consequence of androgen withdrawal per se, since AR activation by androgens can contribute to HIF1 activation in a reversible manner[357,358]. A HIF1 target gene signature is therefore not a reliable hypoxia biomarker in this context. However, since HIF1 signalling seemed to play a major role in the regressive phase of the tumours, the signature might be useful for monitoring ADT effect independent of oxygen status.

In conclusion our experience is that tumours classified as having a greater proportion of hypoxia using these signatures correlated with poorer prognosis in several cancer sites including head and neck, breast, lung and ovarian cancer, in agreement with other methods for identifying hypoxia. The prognostic information provided by hypoxia signatures could allow clinicians and patients to make better informed decisions when planning treatment strategies and this information may be particularly useful in cases where routine histological analysis is unable to provide prognostic value[345]. However, a more powerful application for hypoxia signatures is their use to predict which patients will respond to hypoxia-modifying or hypoxia-targeted treatments (see also Section ''Tumour kinase profiling technology'' below).

Predicting response to hypoxia targeted therapies

The development of a tumour hypoxia gene signature which allows classification of patients for treatment individualisation is a multi-step process. To identify possible candidate genes for a hypoxia gene signature, ideally, hypoxia-responsiveness should initially be verified *in vitro* under simplified and standardised conditions of varying pO_2 conditions. Other micro-environmental conditions, typically co-existing with hypoxia (e.g. low pH), may modify hypoxia-driven changes in gene expression and needs to be taken into account. Given that not all micro-environmental conditions can be mimicked appropriately *in vitro*, additional studies verifying the *in vivo* hypoxia specificity of such candidate genes should also be performed. Finally, clinical testing of the gene-signature in patients should ultimately establish its prognostic value, and importantly, also whether the efficacy of hypoxic intervention may be predicted based on the gene signature. Proteins are the effectors of biological function, but biopsy-based mRNAs are easier to quantify objectively and routinely making them more attractive as clinical biomarkers. In accordance, most gene signatures are RNA-based.

Studies have shown that treatment with nimorazole to sensitise hypoxic cells to radiotherapy improved the disease outcome for head and neck cancer patients[359]. Importantly, the benefit from nimorazole was restricted to patients with relatively high levels of plasma osteopontin, a possible marker of tumour hypoxia Likewise, hypoxic tumours identified by [^{18}F]MISO-PET were better controlled in patients assigned a hypoxia-targeted radio-chemotherapy regimen containing the hypoxic cytotoxin (tirapazamine) than patients assigned to standard radio-chemotherapy[360]. These studies demonstrate the principle that identification of hypoxia and consequential tailoring of treatment strategy could benefit patients with hypoxic tumours. However, another implication highlighted in both of these studies is that patients with low levels of hypoxia did not receive any benefit from the hypoxia modifying/hypoxia targeted intervention, emphasising the importance of sparing patients more intensive and lesser tolerated treatments when these are not

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warranted. Several retrospective studies have shown how hypoxia metagenes can be used to predict treatment response.

Recently, a prognostic and predictive hypoxia gene signature for head and neck cancer patients was developed by Toustrup et al.[361,362]. Initially, a selection of robustly hypoxia-induced genes with little pH-dependency were identified from cell culture studies, using a panel of squamous cell carcinomas cell lines. Interestingly, this initial testing revealed that the hypoxia-driven expression of CAIX, a classical endogenous hypoxia marker, was highly suppressed under low pH conditions, making it questionable as a reliable marker for tissue hypoxia. Next, the *in vivo* hypoxia-specificity of selected pH-independent genes were verified by analysing gene expression profiles in hypoxic versus non-hypoxic tumour tissue dissected from xenograft tumours based on [^{18}F]-FAZA (PET hypoxia tracer) autoradiography of frozen tissue sections. Using a training set of tumour tissue material derived from 58 patients with known hypoxia status, a 15-gene mRNA-based hypoxia classifier was developed. Finally, this classifier was validated in paraffin-embedded biopsy-material from 323 patients with HNSCC randomised for hypoxic modification or placebo in combination with radiotherapy, the DAHANCA 5 study[232]. Tumours categorised as ''more hypoxic'' on the basis of the classifier were associated with a significantly poorer clinical outcome than ''less hypoxic'' tumours. Importantly, outcome in patients with hypoxic tumours was improved and equalised to patients with ''less hypoxic'' tumours by addition of hypoxic modification with the radio-sensitiser nimorazole. The gene signature also revealed that not all patients with hypoxic tumours benefits from this intervention. Specifically, it was demonstrated that tumour hypoxia, as assessed by genetic analysis, were equally distributed among patients with HPV-driven disease and patients with tumours of other etiologies, but that patients with hypoxic HPV-positive tumours did not benefit from hypoxic intervention. This probably relates to the fact that HPV-positive tumour cells are much more sensitive to radiation[363], which may explain their relatively good prognosis and may reduce the potential added value of hypoxia-targeting in this sub-group of cancers. Some of the clinical data are summarised in Figure 9.

This example shows that proper patient characterisation prior to individualised treatment relies on both tumour microenvironment assessment and additional biological testing. In the DAHANCA 5 study, patients only received radiotherapy; however, current state-of-the-art treatment includes chemotherapy. In accordance, as preparation before a potential clinical implementation of the 15-gene hypoxia classifier, current efforts are put into a further validation of the classifier on more cohorts of HNSCC patients treated with radiotherapy/chemoradiotherapy.

Clinical and preclinical studies exploring the value of the 15-gene signature in other cancer types are currently being conducted, and to that end, recently published data by Winther et al.[364] suggests that the gene signature may also be useful for patient stratification in esophagus cancer. On-going preclinical work, focus on evaluation of the gene profile as a reliable marker of hypoxia (and a reliable predictor of the efficacy of hypoxic intervention) in other cancer types including colon and prostate.

Tumour kinase profiling technology

At the molecular level, tumour hypoxia promotes angiogenesis, metastasis, and therapy resistance through the alteration of oxygen-sensitive regulatory mechanisms[41,365]. The adaptive responses to hypoxic stress involve intrinsic activation of a range of signalling pathways mediated by receptor tyrosine kinases such as EGFR, VEGFR and PDGFR family members, collectively contributing to the continuous augmentation of the malignant phenotype[366]. Hence, receptor tyrosine kinase-governed signalling pathways are increasingly recognised as potential biomarkers for stratification of patients to molecularly individualised therapies.

In selecting patients for molecularly targeted agents, both as single-agent therapy and for optimisation of multi-modality cancer treatment, the prevailing gold standards for biomarkers are mainly based on detection of tumour gene aberrations[367]. However, such biomarkers may not be sufficient for the purpose since multiple gene aberrations, which in solid tumours often are the case rather than a single driving gene modification, will affect a wide range of components of the signalling network. Of further note, the plasticity of micro-environmental changes in hypoxic tumours will also contribute to the diversity in signalling activity. Consequently, methodologies comprising the resultant condition of interacting signalling effects may be particularly advantageous to identify functional biomarkers of molecularly targeted therapeutics. Within this frame of reference, kinase substrate array technologies are tools for global profiling of kinase activities in tissue samples without prior knowledge of which signalling pathways are activated, theoretically portraying the state of composite information flow through signalling cascades.

The Tyrosine Kinase PamChip® Array technology (PamGene International B.V., 's-Hertogenbosch, The Netherlands), which our Oslo University Hospital group has applied in studies of tumour biopsies from patients with rectal and prostate cancer and malignant melanoma[368–372], is an array containing 144 peptides, representing 100 different proteins. Each of these kinase targets consists of 13 or 14 amino acids with tyrosine residues for phosphorylation. Substrate phosphorylation levels are measured as signals from a fluorescent anti-phosphotyrosine antibody bound to the phosphorylated peptides. To provide additional information of specific signalling pathways that mechanistically may be important for the biological process of investigation, the tissue lysate can be incubated on the array also in the presence of a selected small-molecular kinase inhibitor for measurement of specific alterations in phosphorylation levels of array substrates. In this manner, the *ex vivo* substrate specificity of the kinase inhibiting agent may also indicate signalling mechanisms that potentially may be actionable tumour targets in patient treatment.

Future prospects on hypoxia detection and imaging

Well-designed studies will need to be conducted to demonstrate the value of hypoxia signatures for patient stratification in a prospective setting. These studies require clear standardisation guidelines for classifying hypoxia scores into high and low groups. In future, hypoxia signatures may become more sophisticated and this will help to derive additional information about tumour biology. For example, signatures that differentiate between acute and chronic hypoxia may be used to determine the contribution of each of these features to specific outcomes including metastasis and treatment response. Another interesting offshoot of this work has been the use of hypoxia signatures to identification novel co-expressed hypoxia-regulated genes. Investigating the role of these newly identified genes in hypoxia biology may reveal new drug targets for modulating the hypoxic response[373].

New therapeutic anti-metastasis treatment

Stereotactic ablative body radiotherapy

Oligometastatic disease is cancer that has spread, but only to one or a small number of sites (classically ≤ 5 metastases in ≤ 3 organs). Although diseases arise in nature, their diagnostic categories are generated by man in ways that are useful to us.

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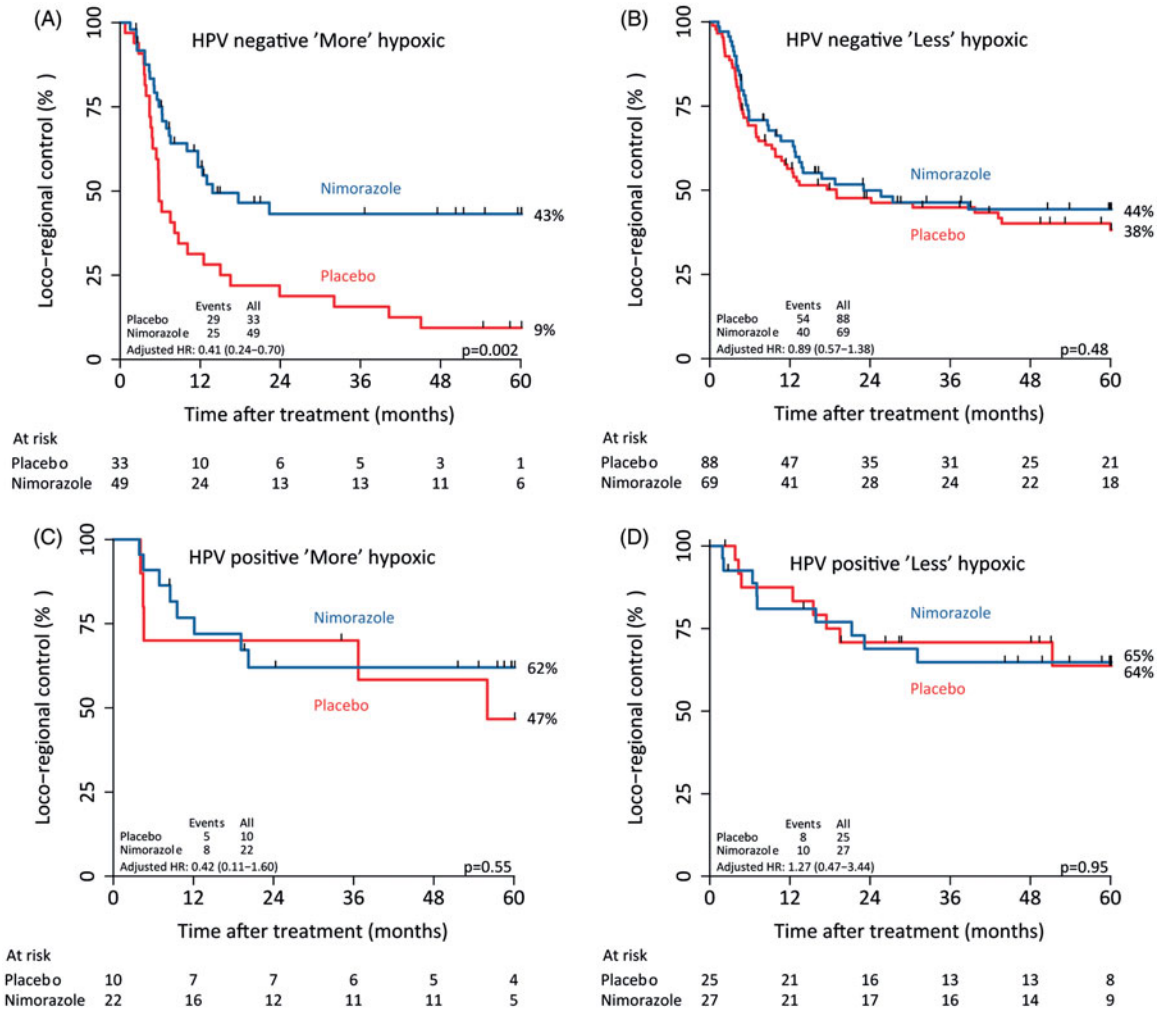

Figure 9. Predictive impact of the 15-gene hypoxia gene signature stratified by HPV status (immunohistochemical detection of p16) on loco-regional control. HPV-negative / ''more'' hypoxic tumours (A), HPV-negative / ''less'' hypoxic tumours (B), HPV-positive / ''more'' hypoxic tumours (C), and HPV-positive / ''less'' hypoxic tumours (D). Loco-regional tumour control is described with the Kaplan–Meier method, compared using the log-rank test, and also expressed as adjusted hazard ratios (HR) using a multi-variate Cox proportional hazards model with the following parameters included: tumour and nodal classification, gender, and age. Modified from Toustrup et al.[362].

Oligometastatic disease is getting more attention because of advances in radiation dose delivery such as by stereotactic body radiation therapy (SBRT) where the radiation dose is delivered with high precision to the tumour.

Stereotactic ablative body radiotherapy (SABR) is a form of high-precision radiotherapy delivering extremely high ablative doses of radiation, usually in 3–8 fractions, combining reproducible patient immobilisation, tumour motion tracking and steep dose gradients, resulting in reduced normal tissue toxicity. SABR achieves excellent local control rates in patients with stage I/II non-small cell lung cancer (NSCLC) and liver metastases of colorectal cancer (CRC)[374]. Nowadays, these favourable results of SABR are being transferred to patients with limited sites of metastatic disease originating from solid tumours (e.g. breast, NSCLC, head and neck, renal cell carcinoma, melanoma, CRC), both at primary diagnosis (synchronous) and during the course of disease[375–382]. Tree et al. reports favourable local control rates of approximately 80% using SABR with few treatment-related side effects[382].

Recently, the MAASTRO group found NSCLC patients with synchronous oligometastases to have a median progression-free survival (PFS) of 12.1 months when treated radically to all known metastatic sites[383]. However, in the vast majority of patients, disease-progression at a distance from the treated site occurs ultimately leading to extensive metastatic disease and cancer-related death. There is a great opportunity to combined modern systemic treatment such as hypoxia targeting drugs with SABR in patients with oligometastasis.

Locally advanced rectal cancer

Despite the introduction of multi-modal therapy for locally advanced rectal cancer (LARC), primarily the combination of surgery and radiation that frequently results in high rates of local control, a substantial number of patients will experience metastatic progression. A rational integration of molecularly targeted agents in combined-modality treatment regimens might cause both improvement of local control in poor-responding patients and reduction in metastasis risk. This strategy, however, will require a clear definition of functional biomarkers for treatment stratification.

Recognising that tumour hypoxia is a common determinant of resistance to cytotoxic therapies and metastatic behaviour, the prospective non-randomised study *Locally Advanced Rectal Cancer—Radiation Response Prediction* of neoadjuvant radiotherapy with concomitant chemotherapy followed by surgery and no further treatment in LARC (ClinicalTrials.gov NCT00278694) offered a unique opportunity to explore this intriguing concept in

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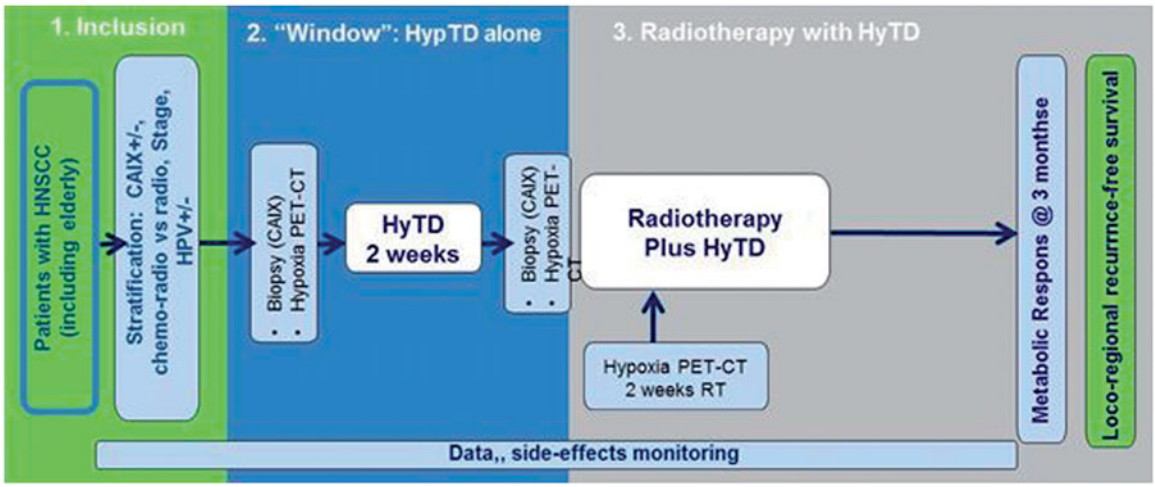

Figure 10. Approach and design of the ''window-of-opportunity'' phase 2 trial with a hypoxia targeting drug combined with a conventional treatment.

a defined clinical context. Utilising the Tyrosine Kinase PamChip® Array technology to analyse study patients' tumour samples, we hypothesised that the study might enable identification of potentially actionable therapeutic targets implicated in hypoxic tumour signalling.

At the time of diagnosis (baseline), tumour biopsy specimens were sampled at rigid proctoscopy from the study patients. Phosphorylation levels of array peptides generated by the tumour samples were correlated to histologic tumour response following neoadjuvant treatment. Essentially, patients with poor response had significantly elevated baseline tumour kinase activity, representing signalling mediated by VEGFR, EGFR and phosphatidylinositol-3-kinase (PI3K), compared to good-responding patients[368]. Next, it appeared that phosphorylation of array peptides representing PDGFR in particular was strongly inhibited by addition of the anti-angiogenic agent sunitinib to the tumour samples from patients without detectable tumour cells in the bone marrow at the time of diagnosis[369]. The recognition that PDGFR-mediated signalling is central for maturation of pericytes during angiogenesis[366] makes it tempting to speculate that intact tumour angiogenic signalling of pericytes is associated with low likelihood of early systemic tumour dissemination in LARC. Of note, in a hypoxic tumour stroma, the vasculature is leaky because of a poorly functioning pericyte layer surrounding the endothelial cells[384,385], which further is associated with metastasis development[386].

Finally, unsupervised clustering analysis of the array phosphosubstrate data separated the tumour samples into two phenotypic populations. The smaller of the groups, displaying high tumour activities within the PI3K signalling network, showed a particularly aggressive disease course as almost a half of cases had developed metastatic disease by less than 1 year of follow-up. Hence, the study indicated that high tumour PI3K-mediated signalling is a biomarker for rapid failure of metastatic disease control in LARC patients following radical treatment of the pelvic cavity[370]. Currently, no consensus exists to whether systemic therapy may reduce the risk of metastatic failure[387,388], which may partly be explained by the paucity of biomarkers for risk assessment and treatment stratification. Given our findings that PI3K may be a key signalling orchestrator both of poor local tumour response to neoadjuvant treatment and importantly, of metastasis development, therapeutic targeting of components of the PI3K complex might be rational to integrate into combined-modality treatment regimens in LARC.

Need for combination treatment

It is was quite clear that to cure a cancer we need to kill several logs of cells and this can best be done by a combination of treatment classically: surgery, radiotherapy, chemotherapy, hormone therapy, targeted drugs and the recent emerging immunotherapy. Interestingly, hypoxia can be an obstacle for all of these six types of treatment. Indeed accumulating evidence implicates the biological responses to hypoxia and the alterations in these pathways in cancer as important contributors to tumourigenesis and treatment efficacy. This has recently prompted several investigations into the possibility of targeting treatment at the biological responses to hypoxia. We then need to rethink strategies to evaluate hypoxia targeting treatment: The classical ''Response Evaluation Criteria In Solid Tumours'' (RECIST) approach would not work because hypoxic cells are proliferating more slowly than well oxygenated cells. Their contribution to tumour growth or in case of specific targeting to tumour regression is limited in the short term.

An interesting approach is the ''window-of-opportunity'' clinical trial, to determine whether this trial design offers a valuable alternative to detect activity of the new therapeutic approaches (Figure 10). The aim is to obtain knowledge about anti-tumour activity of the new therapeutic approaches in a disease state that is not disturbed by previous or simultaneous treatments. The end-point of the window-of-opportunity trial is a clinical end-point, or better an early biomarker of response[389,390].

An example would be to do a hypoxia scan in head and neck cancer followed by a hypoxia targeted drug followed by a second hypoxia scan to see whether the hypoxic fraction is decreased then continue with the classical treatment, e.g. chemo-radiotherapy, with the new agent (Figure 11).

Over the past decade, we have witnessed enormous advances in healthcare. As a result, the delivery of care for oncological patients has been greatly complicated by the rapid expansion of new diagnostic methods and treatment modalities[391]. This evolution has created new challenges such as how to reach evidence level I in view of the ever-diminishing number of seemingly homogenous patients and the explosion of disease and patient parameters[392]. The emergence of individualised medicine contrasts to a certain extent with well-established evidence-based medicine, where randomised trials are designed for selective population[393].

Despite this complexity, individualised cancer treatment is a realistic goal. There is dramatic genetic[394], transcriptomic[395],

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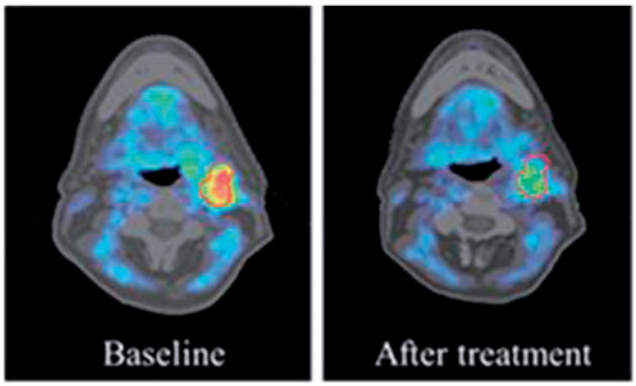

Figure 11. Hypoxia PET before and after hypoxia targeting drug (HyTD).

histological[396] and micro-environmental[397] heterogeneity within individual tumours, and even greater heterogeneity between patients[398]. But if personalised medicine is challenging and necessary, then new techniques and tools are urgently required to aid in clinical decision-making.

The central difficulty is deciding how to integrate diverse, multi-modal information (i.e. clinical, imaging and molecular data) in a transparent and quantitative manner to provide specific clinical predictions that accurately and robustly predict patient outcomes (i.e. generalisable for different patient populations).

Now accurate, externally validated prediction models are being rapidly developed, where multiple features related to the patient's disease are combined into an integrated prediction. The key, however, is standardisation, mainly in data acquisition in all areas, including molecular- and imaging-based assays, patients' preferences and possible treatments. This requires harmonised clinical guidelines, standardised image acquisition and analysis, validated biomarker assay criteria and data-sharing using the identical ontologies. But assessing clinical usefulness is just as important as standardising the development of prediction models with high-quality data, preferably by standardising the design of clinical trials[398]. These crucial steps are the basis of validated decision support systems, which in turn will allow the next steps of ''shared decision making''.

Conclusions (and main results of the consortium)

Specific treatment of hypoxic cells in patients has four fundamental problems which need to be solved. The METOXIA consortium included expertise within all these areas and solutions to these problems have been addressed:

(a) The need of new methods to easily measure oxygenation and/or visualise hypoxic areas both in our pre-clinical models and in patient tumours before and during therapy.

A solution was developed for *in vitro* oxygen monitoring (see Section ''Automated high throughput cell cultivation: fully automated cell culture maintenance'') where the oxygen sensor is moulded into the bottom of the disposable flask so that monitoring of pericellular oxygen for cells attached to the sensor area can be done on line. In animal models and in patients non-invasive methods, such as PET-imaging of the oxygenation status of the tumour have been developed. The most promising new development in this area is the new 2-nitroimidazole nucleoside analogue [^{18}F]-HX4 (see Section ''Diagnostics: hypoxia detection and imaging''). A recent clinical trial in NSCLC patients confirmed that the optimal contrast was obtained 4 h after administration. Research on the use of [^{18}F]FPIMO is on-going with focus on the possibility of labelling pimonidazole in different positions with [^{18}F] to improve tracer stability *in vivo*.

(b) The need of new and more clinically relevant models for pre-clinical testing, particularly models relevant for metastasis.

Both 3D-models using alginate as a scaffold material and 2D methods based on *ex vivo* tumour tissue explants were studied (see Section ''Preclinical cancer models for the assessment of novel therapeutics targeting hypoxia''). Cell migration studies were used as an indicator for ability to metastasize and a first screening of patented compounds were performed using these methods. Animal studies of metastatic capability have been done using an orthotopic model of MDA-MB-231 injected into the mammary fat pad inducing spontaneous metastases in CBA nude mice (see Section ''Models for metastasis'').

(c) The need to identify relevant hypoxia-related patterns of gene expression to improve individualisation of patient treatment.

Low density quantitative real-time polymerase chain reaction (qPCR)-based assays have been developed measuring multiple hypoxia-responsive markers in parallel to identify tumour hypoxia-related patterns of gene expression (hypoxia metagenes) (see Section ''Assessment of tumour hypoxia using hypoxic metagenes'') for classification of patients for treatment individualisation. These methods can even distinguish between acute and protracted (chronic) hypoxia, which are factors of utmost importance regarding response to treatment as well as the ability to metastasize. The strength of the method was demonstrated in a cohort of 2000 breast cancers (see Figure 8).

(d) The need to identify and validate further hypoxia-specific targets essential for tumour growth/metastasis and to synthesise compounds and select potential drugs.

A wide variety of hypoxia-related cell-regulatory processes have been studied. Most emphasis was put on HIF-regulated cascades operating at moderate to weak hypoxia (<1% O_2), and the UPR activated by endoplasmatic reticulum (ER) stress and operating at more severe hypoxia (<0.2%). Both pathways influence expression of several potential targets for drug development, but during the METOXIA project the prioritised targets were the HIF-regulated proteins CAIX[399,400], the lactate transporter MCT4 and the PERK/eIF2α/ATF4-arm of the UPR. Specific inhibition of CAIX and the UPR pathway has been shown to halt tumour metastasis. There are in particular two compound patents which are being followed-up after the end of METOXIA, both relating to inhibition of CAIX. One of these represents compounds able to inactivate CAIX, the other represents compounds with a dual action: These compounds can both inactivate CAIX and act as hypoxic cell radio-sensitisers. For both these types of compounds the development will face the challenge which is common for all treatments specific for hypoxic cells: the compound is insufficient alone to inactivate a tumour. Treatment with the compound will have to be combined with conventional anti-cancer therapies eradicating the aerobic cancer cell population.

Declaration of interest

The authors report no conflicts of interest. The METOXIA project was an EC-financed collaborative project of FP7 (HEALTH-F2-2009-222741) named ''Metastatic tumours facilitated by hypoxic tumour micro-environments'' which ended by 31 July 2014.

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