Targeting hypoxia in cancer therapy

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Abstract | Hypoxia is a feature of most tumours, albeit with variable incidence and severity within a given patient population. It is a negative prognostic and predictive factor owing to its multiple contributions to chemoresistance, radioresistance, angiogenesis, vasculogenesis, invasiveness, metastasis, resistance to cell death, altered metabolism and genomic instability. Given its central role in tumour progression and resistance to therapy, tumour hypoxia might well be considered the best validated target that has yet to be exploited in oncology. However, despite an explosion of information on hypoxia, there are still major questions to be addressed if the long-standing goal of exploiting tumour hypoxia is to be realized. Here, we review the two main approaches, namely bioreductive prodrugs and inhibitors of molecular targets upon which hypoxic cell survival depends. We address the particular challenges and opportunities these overlapping strategies present, and discuss the central importance of emerging diagnostic tools for patient stratification in targeting hypoxia.

Hypoxia influences many aspects of the biology of tumours and their responses to therapy. Initially, hypoxia arises because of oxygen diffusion limitations in avascular primary tumours or their metastases, but the tumour microvasculature (induced in part as a response to this hypoxia) is highly abnormal[1,2] and often fails to rectify the oxygen deficit. This persistent hypoxia reflects the spatial disorganization of tumour vascular networks, leading to intercapillary distances that are often beyond the diffusion range of oxygen (which is up to ~200 μm, depending on the local oxygen concentration in blood plasma). In addition to this diffusion-limited hypoxia, temporally unstable blood flow in tumour microvascular networks also leads to fluctuating perfusion-limited hypoxia[3].

The many effects of hypoxia on tumour biology include: selection of genotypes favouring survival under hypoxia–re-oxygenation injury (such as *TP53* mutations[4]); pro-survival changes in gene expression that suppress apoptosis[5] and support autophagy[6]; and the anabolic switch in central metabolism[7]. Hypoxia also enhances receptor tyrosine kinase-mediated signalling[8], tumour angiogenesis[9], vasculogenesis[10], the epithelial-to-mesenchymal transition[11], invasiveness[12] and metastasis[13], as well as suppressing immune reactivity[14]. In addition, hypoxia contributes to loss of genomic stability through the increased generation of reactive oxygen species (ROS)[15] and the downregulation of DNA repair pathways[16].

In part because of these effects on tumour development, hypoxia is implicated in resistance to therapy through multiple mechanisms (shown for cytotoxic agents in TABLE 1; see also Supplementary information S1 (tables)). Reflecting these major roles in cancer biology and therapy, there is compelling evidence that hypoxia can compromise clinical outcomes in human cancer (TABLE 2). However, as noted in TABLE 1, some changes in hypoxic cells can result in increased drug sensitivity; these exceptions caution against the frequent generalization in the literature that hypoxic cells are invariably chemoresistant.

The apparent extent of hypoxia in human tumours depends on the methods used to detect it; the most widely used methods are indicated in TABLE 2. Invasive oxygen electrodes provide the most direct measure and demonstrate extreme heterogeneity of oxygenation within and between tumours in every tumour type evaluated in patients[17]. Increasingly, evaluation of hypoxia in the clinic is shifting to the monitoring of endogenous markers, especially the transcriptional targets of the hypoxia-inducible factors (HIFs), and exogenous 2-nitroimidazole probes, such as pimonidazole, that bind covalently to SH-containing molecules (thiols) in hypoxic tissue[18,19]. The use of these markers to image hypoxia in a human tumour is illustrated in FIG. 1a, which shows the typically more restricted distribution of bound pimonidazole than the HIF1 target carbonic anhydrase 9 (CA9). This and other evidence indicates that metabolic activation of 2-nitroimidazole probes requires more severe hypoxia than does the HIF1 response. Quantitative understanding of hypoxia in tumours (and physiological hypoxia in some normal tissues) is far from complete, but the oxygen concentration dependencies

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At a glance

- Hypoxia represents a compelling therapeutic target, given that it has a major role in tumour development and resistance to therapy, and that the levels of hypoxia are more severe in most tumours than normal tissues.
- One approach to targeting hypoxia seeks to develop bioreductive prodrugs that are activated by enzymatic reduction in hypoxic tissue. These prodrugs are chemically diverse and represent two distinct strategies: activation under moderate hypoxia (as exemplified by tirapazamine) or only under severe hypoxia (as exemplified by PR-104). In the latter case, diffusion of the active drug to less hypoxic cells is essential.
- A second approach seeks small molecule inhibitors against molecular targets involved in the survival of hypoxic cells. Current interest focuses on the inhibition of the hypoxia-inducible factor 1 (HIF1), the unfolded protein response (UPR) and mTOR pathways, but the most important vulnerabilities in hypoxic cells are not well defined. Most molecularly targeted agents have been 'repurposed' from other applications, and have low selectivity as hypoxic cytotoxins.
- Both approaches face substantial challenges in relation to off-target effects, which, ironically, also present opportunities. For bioreductive prodrugs, activation by aerobic reductases can contribute to normal tissue toxicity, but this is exploitable in tumours that highly express these enzymes. For molecularly targeted agents, hypoxia-independent signalling through the same pathways may provide opportunities for additional antitumour activity.
- Both bioreductive prodrugs and molecularly targeted agents also need to overcome the problem of drug penetration through poorly perfused hypoxic tissue; strategies for addressing this requirement are being developed.
- The current generation of bioreductive prodrugs generate DNA-reactive cytotoxins, making them difficult to combine with conventional chemotherapy because of overlapping toxicity. This challenge is stimulating the development of bioreductive prodrugs that release molecularly targeted agents as their effectors, potentially combining the best features of both approaches.
- Given the marked heterogeneity in hypoxia between tumours of the same type, the clinical exploitation of hypoxia using all of these approaches will require their co-development with companion diagnostics for hypoxia (and for other determinants of sensitivity).

for some of the critical biological processes considered in this Review are illustrated schematically in FIG. 1b. These differences in oxygen concentration thresholds have important implications for targeting hypoxic cells, as have differences in the spatial distribution and duration of hypoxia and the genetic and environmental context in which hypoxia occurs. In particular, these factors will dictate the choice of hypoxia-targeted therapy that best complements existing agents used to treat the oxic cell population in tumours.

The compelling evidence for hypoxia in tumour tissue and its therapeutic importance makes hypoxia a high priority target for cancer therapy. In this Review we describe recent progress in developing small molecule drugs to kill hypoxic cells, including bioreductive prodrugs that are activated selectively under hypoxia, and drugs that inhibit molecular targets in hypoxic cells. We focus here on agents that kill hypoxic cells directly, rather than inhibitors of hypoxia-dependent processes such as angiogenesis.

Bioreductive prodrugs
Biologically inactive molecules that are converted to an active drug by enzymatic reduction.

Superoxide
A free radical formed by a one-electron reduction of oxygen, including by electron transfer from a prodrug free radical. Despite its name, superoxide itself is not highly reactive and is generally less toxic than the reduced prodrug, so its generation represents a detoxification mechanism in aerobic cells.

Bioreductive prodrugs

Chemical classes and mechanisms of action. The concept of activating prodrugs selectively in tumours, to achieve targeted delivery of cytotoxins, has a long history. The first clear demonstration was the reactivation of β-glucuronide metabolites of an aniline nitrogen mustard in tumours with high β-glucuronidase activity[20], but such approaches have struggled with the challenge of finding tumours with high enough expression of the activating enzymes to achieve useful selectivity. Hypoxia is potentially a more generic feature, with a clear basis for tumour selectivity, although expression of the activating enzymes is also critically important in this context.

Five different chemical moieties (nitro groups, quinones, aromatic *N*-oxides, aliphatic *N*-oxides and transition metals) have the potential to be metabolized by enzymatic reduction under hypoxic conditions, and thus provide the basis for the design of bioreductive prodrugs for exploiting tumour hypoxia. The mechanisms by which bioreductive prodrugs are selective for hypoxic cells are summarized in FIG. 2A; most often these mechanisms involve the re-oxidation by oxygen of the initial free radical intermediate formed by a one-electron reduction of the prodrug, thus generating superoxide. This futile redox cycling ensures that steady-state concentrations of the prodrug radical are kept low in oxic cells, resulting in hypoxia-selective cell killing provided that the prodrug radical (or its downstream products) is more cytotoxic than superoxide or the unreduced prodrug.

Inhibition of drug reduction by oxygen through this redox cycling mechanism was first demonstrated for nitro compounds[21] and was subsequently shown to be responsible for the hypoxia-selective cytotoxicity of nitroimidazoles[22]. This bioreductive mechanism is distinct from hypoxic cell radiosensitization by the same compounds[23], which is due to the ability of these compounds to replace oxygen in oxidizing ionizing radiation-induced DNA free radicals to generate cytotoxic DNA strand breaks[24]. This first proof-of-principle demonstration of the hypoxia-selective cytotoxicity of bioreductive prodrug activity stimulated the search for ways of linking nitroreduction to the formation of more potent cytotoxins, illustrated by PR-104 and TH-302 (FIG. 2B), and for other redox moieties capable of hypoxia-selective metabolic activation.

The potential for using quinones in this context can be traced to the discovery in the 1960s that the DNA-crosslinking anticancer antibiotic mitomycin C is activated by reduction of its indoloquinone moiety[25,26]. Sartorelli's group subsequently designed simpler quinone bioreductive alkylating agents[27], which were proposed to exploit the more reducing environment in tumours relative to normal tissues[28]. It was later shown that the bioreductive activation of quinones occurs selectively under hypoxia[29] through a redox cycling mechanism[30] analogous to that for nitro compounds, but with two sequential one-electron reductions (first to the semiquinone and then to the hydroquinone).

Subsequently, three other chemical moieties capable of hypoxia-selective metabolic reduction by tumour cells have been discovered. Martin Brown[31] showed that the aromatic *N*-oxide tirapazamine (TPZ; FIG. 2B) is 50–200-fold more toxic to hypoxic than oxic cells in culture[31] owing to one-electron reduction to a DNA-damaging free radical (originally thought to be the TPZ radical itself, but now considered to be an

Table 1 | **Mechanisms of resistance (and sensitivity) of hypoxic cells to cytotoxic therapy***

Effect of hypoxia	Resistance or sensitivity?	Mechanism	Agents affected	Example
Lack of oxidation of DNA free radicals by O_2	Resistance	Failure to induce DNA breaks	Ionizing radiation	2–3-fold increase in ionizing radiation dose required for equivalent cell kill
			Antibiotics that induce DNA breaks	Bleomycin
Cell cycle arrest in G1 or G2 phase	Resistance	Repair before progression to S or M phase	Cycle-selective chemotherapy drugs	5-Fluorouracil
Cell cycle arrest in S phase	Sensitivity	Collapse of stalled replication forks	PARP inhibitors‡	Veliparib (ABT-888)
Distance from vasculature (indirect)	Resistance	Compromised drug exposure	Drugs extensively bound in tumour cells	Taxanes
Extracellular acidification (indirect)	Resistance	Decreased uptake	Basic drugs	Doxorubicin
	Sensitivity	Increased uptake	Acidic drugs	Chlorambucil
Resistance to apoptosis	Resistance	Genetic selection of *TP53* mutants	Multiple	
		Downregulation of BID and BAX	Multiple	Etoposide
Genomic instability	Resistance	Mutagenesis	Multiple	*DHFR* amplification and methotrexate
Suppression of DNA repair	Resistance	Downregulation of MMR	DNA methylating agents	
	Sensitivity	Downregulation of NER	Bulky DNA monoalkylating and crosslinking agents	
		Downregulation of HR	DNA crosslinking agents	Cisplatin
HIF1 stabilization	Resistance	Expression of ABC transporters	ABC transporter substrates	MDR1 and doxorubicin
		Downregulation of NHEJ	Agents that induce DSBs	Etoposide

BAX, BCL2-associated X protein; BID, BH3 interacting domain death agonist; *DHFR*, dihydrofolate reductase; DSB, double strand break; HIF1, hypoxia-inducible factor 1; HR, homologous recombination; MDR1, multidrug resistance protein 1; MMR, mismatch repair; NER, nucleotide excision repair; NHEJ, non-homologous end joining; PARP, poly(ADP-ribose) polymerase. *See also Supplementary information S1 (tables) for tables with references. ‡Also sensitized by downregulation of HR under hypoxia.

oxidizing hydroxyl[32] or benzotriazinyl[33] radical arising spontaneously from the TPZ radical) (FIG. 2B). Later, Laurence Patterson[34] and ourselves[35] independently demonstrated that inhibition by oxygen of the bioreduction of aliphatic *N*-oxides to the corresponding tertiary amines can also be used as a basis for hypoxia-activated prodrugs, in these examples through increasing DNA binding affinity of intercalators (illustrated for banoxantrone (also known as AQ4N) in FIG. 2B). For the aliphatic *N*-oxides, hypoxic selectivity stems from inhibition of two-electron reductases by oxygen (FIG. 2A), rather than redox cycling. Examples of the fifth class (transition metals) include cobalt(III)[36,37] and copper(II)[38] complexes capable of hypoxia-selective bioreductive activation through one-electron reductions of the metal centres to unstable cobalt(II) or copper(I) complexes that then dissociate to release cytotoxic ligands.

Bioreductive prodrugs under recent or ongoing clinical development (FIG. 3; TABLE 3) include examples of each of these chemotypes (except transition metal complexes, for which hypoxic cell killing has only been reported in cell culture). Other than TPZ and apaziquone (also known as E09), for which Phase III clinical trial results are pending, the compound currently most advanced in clinical testing is TH-302 (FIG. 2B). This 2-nitroimidazole-based nitrogen mustard prodrug has shown promising activity in a Phase I study[39] and is being evaluated in multiple Phase I and II trials, including a randomized Phase II trial with gemcitabine in pancreatic cancer (www.ClinicalTrials.gov identifier NCT01144455). The clinical status of the other compounds is discussed below in relation to unique features of their mechanisms of action. These prodrugs illustrate diverse strategies for exploiting oxygen-sensitive biotransformations to achieve cytotoxic activation (FIG. 2B), and are representative of other prodrugs reviewed previously[40–43]. The prodrugs also differ in their quantitative oxygen dependence (K_{O2}, the K_i for inhibition by oxygen), the activating reductases and the nature of the resulting DNA lesions (TABLE 3). A recent addition is a chloromethylbenzindoline prodrug, SN29730, which generates a potent DNA minor groove alkylator on nitroreduction and has high hypoxic potency and selectivity *in vitro* and *in vivo*[44]. A common feature of all these prodrugs is that interference with the DNA replication fork appears to be the main mechanism of cytotoxicity, as illustrated by the dependence of the hypoxic cytotoxicity of TPZ[45] — and the alcohol metabolite of PR-104, PR-104A[46] — on homologous recombination (HR) repair, which is required for the resolution of damage at the replication fork[47].

Replication fork
The branch-point structure that forms between two DNA template strands during DNA replication at which nascent DNA synthesis is ongoing.

Homologous recombination
(HR). High-fidelity repair of DNA lesions, including double-strand breaks, in S and G2 phases of the cell cycle, using a sister chromatid as a template.

Identifying and exploiting the activating reductases. Targeting hypoxia with bioreductive prodrugs depends on tumour expression of the appropriate activating reductases. Most of the one-electron reductases responsible for the redox cycling (and hence the hypoxic selectivity) of prodrugs appear to be NAD(P)H-dependent flavoproteins with low substrate affinities and specificities as xenobiotic metabolizing enzymes; their identification represents an important ongoing challenge (BOX 1).

Reductases that catalyse concerted two-electron reductions provide an alternative pathway for bioreductive prodrug activation (FIG. 2A) and represent both an opportunity and challenge for tumour targeting. These enzymes fall into two broad groups. Haemoproteins, such as cytochrome P450s (CYPs), especially CYP3A4, can catalyse the two-electron reduction of AQ4N[48]. A recently identified extrahepatic CYP, CYP2S1, also reduces AQ4N[49], which is notable given that this enzyme is upregulated by HIF1 (REF. 50). The one-electron reductase inducible nitric oxide synthase (iNOS; also known as NOS2) is also upregulated under hypoxia (BOX 1), and can similarly catalyse the two-electron reduction of AQ4N through its CYP-like haem domain[51]. Importantly, although these haem-dependent reductions of *N*-oxides do not generate an oxygen-sensitive radical intermediate, they are nonetheless inhibited by oxygen[49,51], presumably through competitive binding of O_2 and the *N*-oxide to the haem prosthetic group. This process is therefore potentially exploitable for targeting hypoxia, although the K_{O2} is not well defined, and whether this pathway is fully suppressed under oxic conditions is unclear.

A second group of two-electron reductases catalyse hydride (H^-) transfer from NAD(P)H and are not inhibited by oxygen. These can bypass the oxygen-sensitive free radical intermediate during reduction of quinones, nitro compounds and aromatic *N*-oxides. The best studied enzyme of this class is NAD(P)H dehydrogenase [quinone] 1 (NQO1; also known as DT-diaphorase), which catalyses the facile two-electron reduction of quinones including apaziquone and the aziridinylbenzoquinone RH1 to their hydroquinones[52]. NQO1 also reduces the dinitrobenzamide CB 1954 (tretazicar) to its active 4-hydroxylamine metabolite[53]. Although CB 1954 is a poor substrate for human NQO1, it is efficiently reduced by its paralogue NQO2 using dihydronicotinamide riboside (NRH) as a cofactor[54]. NQO2 also catalyses aerobic reduction of RH1 (REF. 55). In addition, the NADH-dependent two-electron reductase aldo–keto reductase 1C3 (AKR1C3) has recently been shown to reduce PR-104A (but not other bioreductive prodrugs) in some human tumour cell lines under aerobic conditions[56].

Aerobic two electron reductions by these enzymes represent 'off-target' activation in the context of hypoxia and are likely to contribute to the normal tissue toxicity of some quinones and nitro compounds, as illustrated by the resistance of *Nqo1* knockout mice to mitomycin C-induced myelotoxicity[57] and the expression of NQO1 in many normal human tissues[58]. However, this activation may also be therapeutically exploitable in tumours that highly express these enzymes. *NQO1*, *NQO2* (REF. 59) and *AKR1C3* (REFS 56,60) are each transcriptionally regulated, through their antioxidant response elements (AREs), by the transcription factor nuclear

Table 2 | **Representative examples of the prognostic and predictive significance of hypoxia in human cancer***

Measure of hypoxia	Probe	Clinical setting	Outcome for hypoxic tumours
Oxygen concentration	Eppendorf oxygen electrode	Chemoradiation of advanced HNSCC	Worse OS
		Radiotherapy of soft tissue sarcomas before surgery	Worse DFS owing to a higher rate of distant metastasis
		Brachytherapy of localized prostate cancer	Decreased biochemical control (shown by PSA levels)
		Cervical carcinoma	Worse DFS in node-negative patients owing to a higher rate of distant metastases
Endogenous markers	HIF1α	Node-negative breast cancer	Worse OS
	HIF1α	BRCA1 mutant breast cancer	Worse DFS
	HIF2α, CA9	CHART trial in HNSCC	Worse local control and OS
	CA9	Adjuvant chemotherapy of breast cancer	Worse OS
	Osteopontin	Radiotherapy for HNSCC	Nimorazole (hypoxic radiosensitizer) improved local control and OS
	Lysyl oxidase	Breast cancer	Worse metastasis-free survival
	Hypoxic gene signature	HNSCC and breast cancer	Worse outcome, multiple end points
	Hypoxic gene signature	Hepatocellular carcinoma	Worse OS
Exogenous probes	Pimonidazole	Radiotherapy for advanced HNSCC	Worse local control
	EF5	Post-surgical radiotherapy of HNSCC	Worse DFS

CA9, carbonic anhydrase 9; CHART, continuous hyperfractionated accelerated radiotherapy; DFS, disease-free survival; EF5, etanidazole pentafluoride; HIF, hypoxia-inducible factor; HNSCC, head and neck squamous cell carcinoma; OS, overall survival; PSA, prostate specific antigen. *See also Supplementary information S1 (tables) for tables with references.

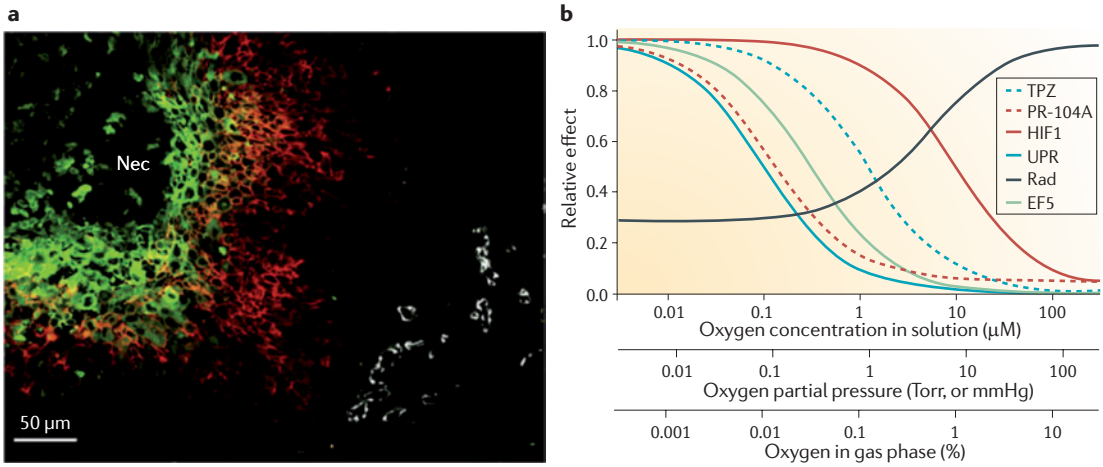


Figure 1 | **Oxygen dependence of hypoxia-responsive processes in tumours. a** | Pseudocolour immunofluorescence showing the difference in distribution of covalently bound pimonidazole (green), an exogenous 2-nitroimidazole hypoxia marker, and hypoxia-inducible factor 1 (HIF1)-regulated carbonic anhydrase 9 (CA9; red), an endogenous marker of hypoxia. This distribution is shown relative to blood vessels (white) and necrosis (Nec) in a representative region of a human squamous cell carcinoma of the larynx. **b** | Schematic representation of quantitative oxygen dependencies for ionizing radiation, bioreductive activation of prodrugs and imaging agents, and biological responses to hypoxia. Three commonly used units for oxygen concentration are shown on the *x* axis, assuming that the culture medium is in equilibrium with humidified gas mixtures at atmospheric pressure[77]. The curves are based on representative oxygen sensitivity parameters for clonogenic cell killing by: ionizing radiation (Rad)[185], tirapazamine (TPZ)[78] and PR-104A[83]. Also shown is binding of the 2-nitroimidazole etanidazole pentafluoride (EF5) to intracellular proteins[186]. Biological responses to hypoxia are time- and cell-type-dependent; the indicative relationships shown here are based on acute stabilization of HIF1 in HT1080 cells[186] and evidence that the unfolded protein response (UPR) is rapidly induced only under severe hypoxia[110,187]. Part **a** is reproduced, with permission, from REF. 150 © (2009) Elsevier Science.

factor erythroid 2-related factor 2 (NRF2; also known as NFE2L2). NRF2, in turn, is controlled by a redox-sensitive cytoplasmic repressor Kelch-like ECH-associated protein 1 (KEAP1), and independently by PRKR-like endoplasmic reticulum kinase (PERK; also known as eIF2AK3)[61]. Both of these signalling pathways provide the potential for indirect upregulation of NRF2-regulated reductases under hypoxia through increased ROS (especially under conditions of fluctuating hypoxia), leading to KEAP1 inactivation or activation of unfolded protein response (UPR) signalling through PERK (see below). High expression of NQO1 is the major driver for clinical development of apaziquone as an intravesicular (topical) therapy for non-invasive bladder cancer[62], and RH1 is also being explored for treatment of tumours with high NQO1 expression[63]. The combination of CB 1954 with the synthetic reducing cofactor caricotamide (also known as EP-0152R), an NRH analogue, has recently been explored for the treatment of NQO2-expressing hepatocellular carcinomas (HCCs). Similarly, high expression of AKR1C3 in some non-small-cell lung cancers and HCCs[56] has led to pilot clinical studies of PR-104 in these cancers, and evaluation is ongoing for acute myeloid leukaemia (AML), based on the high expression of *AKR1C3* mRNA in leukaemic cells from some patients with AML[64]. In each case, the additional hypoxia-selective activation by one-electron reductases is potentially beneficial, including in leukaemias and multiple myeloma, given recent evidence for hypoxia secondary to their expansion in the bone marrow[65,66].

TPZ is also a substrate for NQO1, but uniquely sidesteps the complications of two-electron reduction in that its mono-oxide and non-oxide reduction products (X and Y in FIG. 2A) are relatively non-toxic[67]. This attractive feature of the aromatic *N*-oxides is retained in second-generation TPZ analogues such as SN30000 (REF. 68).

Bioreductive prodrug micropharmacokinetics: the extravascular transport problem. Limited extravascular penetration of drugs, an important contributor to the chemoresistance of solid tumours[69], becomes more crucial when the target cells are confined to hypoxic zones distant from functional blood vessels. The problem is particularly severe for bioreductive prodrugs, given that they are designed to be metabolized as they diffuse into hypoxic zones; if this metabolism is too facile, exposure of the most hypoxic cells will inevitably be compromised. This probably underlies the much lower hypoxic selectivity of TPZ in tumours than in low-density cell cultures[70]. The first suggestion that metabolic consumption of TPZ compromises its tissue penetration came from studies showing loss of activity in hypoxic multicellular spheroids[71]. This was confirmed in more quantitative studies[72,73] using another three-dimensional cell culture model, multicellular layers (MCLs), a model that is more amenable to the direct measurement of drug diffusion.

The importance of prodrug penetration in determining hypoxic cell killing in tumours is illustrated by a comparison of 15 TPZ analogues with widely different extravascular transport properties[74]. In this study the

Multicellular spheroids
Spherical clusters of cells that grow large enough to become diffusion-limited, and thus model some features of the tumour microenvironment.

Multicellular layers
(MCLs). Three-dimensional cell cultures that model the extravascular compartment of tumours. Grown on collagen-coated micro-porous membranes, they allow measurement of drug diffusion and metabolism in tumour-like tissue.

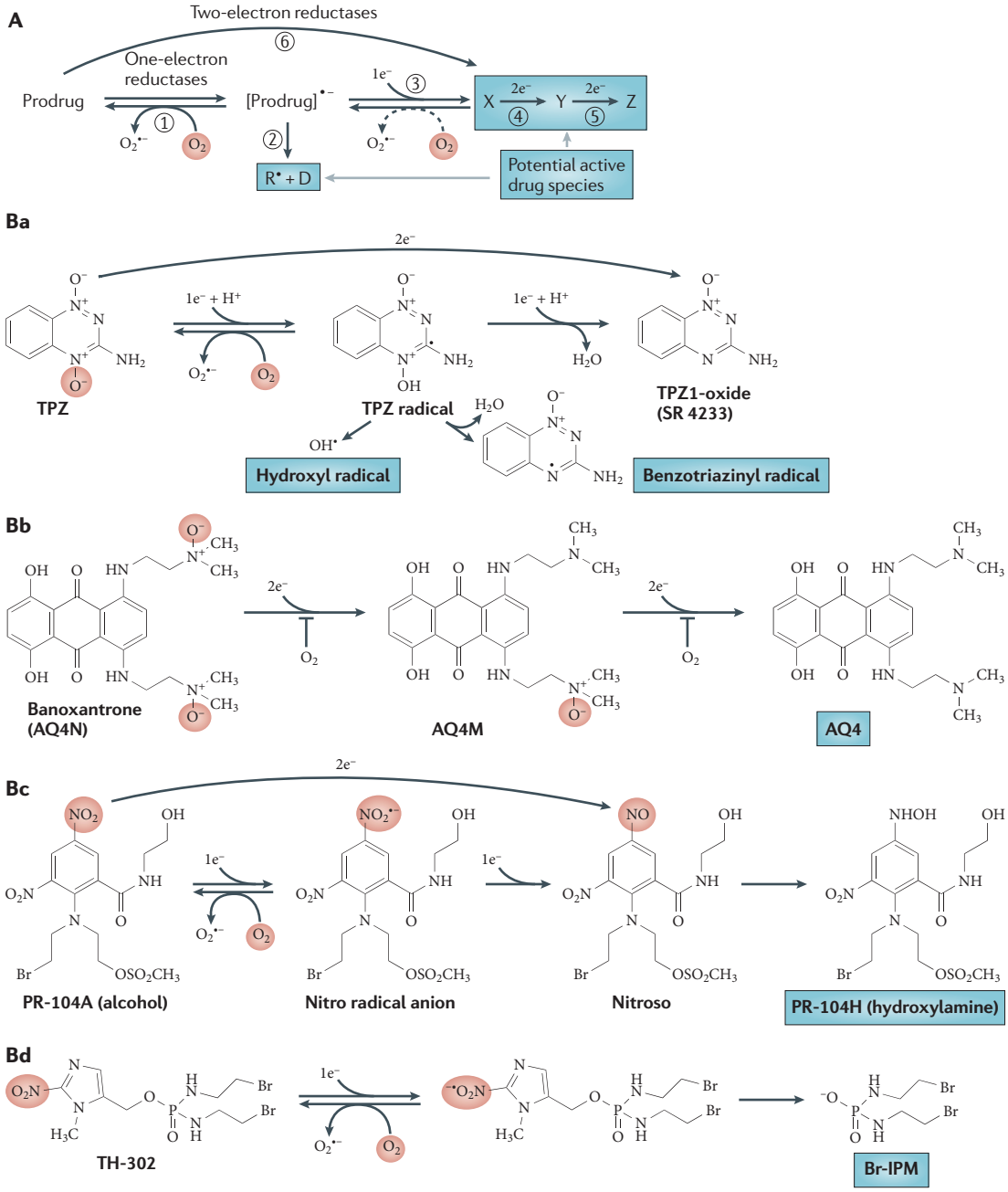


Figure 2 | **Mechanisms of metabolic activation of bioreductive prodrugs.** The cytotoxic metabolites are shown in blue. **A** | Generalized scheme showing competing one-electron and two-electron reductions of prodrugs. One-electron reduction generates a prodrug radical that can be re-oxidized by oxygen (reaction 1) in oxic cells, but generates active drug (blue boxes) in hypoxic cells, either by fragmentation of the prodrug radical (reaction 2) or by its further reduction, usually by disproportionation (reaction 3) and subsequent reduction of the two electron reduction product, X (reactions 4 and 5). Some prodrugs are also reduced by a concerted two-electron reduction (reaction 6), thus bypassing the oxygen-sensitive prodrug radical. Two-electron reduction is typically insensitive to oxygen, with important exceptions (see main text). **B** | Examples of well-studied prodrugs that exploit bioreduction in different ways to elicit selective killing of hypoxic cells. **Ba** | Reduction of an aromatic *N*-oxide to generate a DNA-reactive free radical; **Bb** | reduction of an aliphatic *N*-oxide to unmask a DNA intercalator; **Bc** | nitroreduction as an electronic switch to activate a reactive centre, thus generating an activated nitrogen mustard; and **Bd** | nitroreduction to initiate fragmentation to a non-radical cytotoxin, such as a nitrogen mustard.

tissue diffusion coefficient and bioreductive metabolism kinetics of each prodrug was measured using MCLs grown from HT29 human colon adenocarcinoma cells. These measurements were used to develop a spatially resolved pharmacokinetic and pharmacodynamic model describing pharmacokinetics (concentration–time profiles) and pharmacodynamics (cell killing probability) as a function of position in a tumour microvascular network. Hypoxic

Bystander effect
In the context of bioreductive prodrugs, the killing of adjacent cells that lack prodrug-activating ability through local diffusion of the active drug.

cell killing in HT29 tumour xenografts was well predicted by the model, but only when extravascular transport was included explicitly. This study demonstrated that prodrug reduction kinetics need to be optimized to balance the competing requirements of metabolic stability (for maximal tissue penetration) and metabolism to the cytotoxic metabolite (for maximal cytotoxicity in hypoxic cells).

Until recently the penetration problem has largely been ignored during the development of bioreductive prodrugs, many of which have been found to lack activity as hypoxic cytotoxins in xenograft models despite marked hypoxic selectivity in low-density cell cultures. Some progress has been made in defining the physicochemical properties (such as lipophilicity, molecular weight and hydrogen bond donors and acceptors) that determine diffusion coefficients using MCLs, at least for TPZ analogues[75]. This has assisted the design of new analogues with higher tissue diffusion coefficients, making it possible to accommodate higher rates of bioreductive metabolism without compromising penetration[76]. These features are illustrated by SN30000 (TABLE 3), which has higher activity than TPZ against hypoxic cells in multiple xenograft models[68].

Finessing bioreductive prodrug activation:* K *values and bystander effects. Bioreductive prodrugs can act as direct oxygen sensors through redox cycling or other mechanisms of reductase inhibition by oxygen, as outlined above. However, their quantitative oxygen dependence is crucially important for their ability to complement other anticancer agents such as ionizing radiation (FIG. 1b), and differs among prodrugs.

The elimination of hypoxic tumour cells at 'intermediate' oxygen concentrations (~1–10 μM oxygen) is arguably more important than the most severely hypoxic or anoxic cells, which are less frequent and probably less likely to contribute to tumour regrowth after therapy. Two different bioreductive prodrug strategies are being explored for targeting these moderately hypoxic cells, each with different strengths and weaknesses. One strategy is to use prodrugs with relatively high K_{O2} to provide activation under moderate hypoxia. The only bioreductive prodrugs demonstrated to be activated under such conditions are TPZ[77,78] and its analogues, such as SN30000 (REF. 68), which have K_{O2} values of ~1 μM in cell culture (TABLE 3).

The other strategy is to confine prodrug activation to more severely hypoxic cells (K_{O2} ~0.1 μM), which has the advantage of restricting activation to pathologically hypoxic regions in tumours and thus avoiding activation under physiological hypoxia in normal tissues. This also limits the metabolic loss of prodrugs during diffusion into hypoxic zones. These very low K_{O2} values — although difficult to measure experimentally because of technical limitations in controlling and quantifying low oxygen concentrations in respiring cell cultures — seem to be typical of quinones[79], nitro compounds[80] and cobalt complexes[81]. These bioreductive prodrugs can be expected to spare many radioresistant and chemoresistant hypoxic cells at oxygen concentrations above the drugs' K_{O2}. In this case it may be crucially important that the active bioreductive metabolites can diffuse to cells at higher pO_2 (known as the bystander effect). Such local diffusion has been demonstrated for CB 1954 and dinitrobenzamide mustards using anoxic MCL co-cultures in which 'activator' cells overexpressing NADPH–cytochrome P450 reductase (CYPOR; also known as POR) facilitate the killing of 'target' cells that are less able to activate the prodrugs[82]. PR-104A provides an example of a bioreductive prodrug with this profile (a low K_{O2} and efficient bystander killing)[83]. Which of these strategies (high K_{O2} versus low K_{O2} plus bystander effect) is preferable may depend on tumour-specific features such as the depth and spatial distribution of hypoxia (for example, whether most moderately hypoxic cells are contiguous with more severely hypoxic cells) and on treatment-specific features such as the oxygen dependence and extravascular penetration of any other agents used in combination.

Beyond DNA-reactive cytotoxins as effectors for bioreductive prodrugs. A common feature of all bioreductive prodrugs currently in development (TABLE 3) is that their active metabolites are DNA-reactive cytotoxins that damage the replication fork. Although the DNA replication fork can be considered the most successful chemotherapy target to date[84], toxicity to proliferating normal tissues is an inescapable consequence. Existing chemotherapy and chemoradiation protocols are already titrated to maximal myelotoxicity, which limits the opportunities to add the current generation of bioreductive prodrugs to standard therapies. This makes it attractive to consider adapting bioreductive prodrug design to release a broader range of active metabolites, including non-genotoxic inhibitors of molecular targets. Early examples were 2-nitroimidazole prodrugs that, on chemical reduction, release the poly(ADP-ribose) polymerase 1 (PARP1) inhibitor 5-bromoisoquinolone[85] and the prototypical cyclooxygenase inhibitor aspirin[86]. More recently a similar approach has been used to release the tubulin-stabilizing drug combretastatin A4 (REF. 87) and the lysyl oxidase inhibitor β-aminoproprionitrile by bioreduction of prodrugs under hypoxia[88]. In addition, quaternary ammonium nitroheterocyclic bioreductive triggers[89] have been used to release non-myelotoxic, irreversible pan-ERBB inhibitors under hypoxia[90]. The prototype of this new class, SN29966, provides marked activity as a monotherapy against human tumour xenografts, a result that is suggested to reflect the ability of this prodrug to exploit fluctuating hypoxia because of its long residence time in tumours[90].

Molecular targets in hypoxic cells

The identification of molecular mechanisms that mediate cellular responses to hypoxia has stimulated interest in targets that might compromise the survival of hypoxic cells if inhibited. The two main oxygen-responsive signalling pathways that mediate adaptation to hypoxia are centred on the HIF family of transcription factors[3,91,92] and the UPR[93], whereas mTOR presents a less well-defined opportunity to target hypoxic cell survival (FIG. 4).

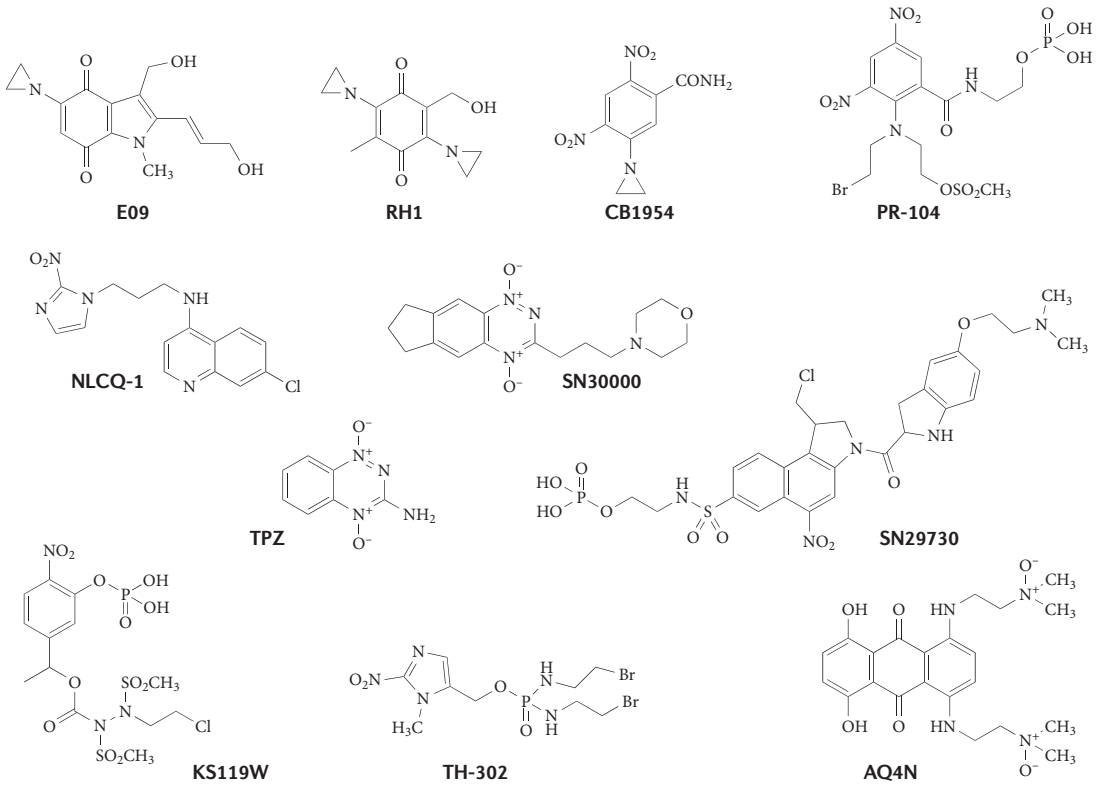


Figure 3 | **Structures of bioreductive prodrugs.** Structures of the prodrugs presented in TABLE 3 and in the main text are shown.

HIFs. Regulation of HIF1α and HIF2α (also known as EPAS1) by oxygen-dependent dioxygenases such as prolyl hydroxylase domain (PHD) enzymes, the primary oxygen sensors, leads to a broad, adaptive response to hypoxia. This response includes the transcription of genes involved in angiogenesis (such as vascular endothelial growth factor A (*VEGFA*)), metabolic adaption (such as *SLC2A1*, which encodes the glucose transporter GLUT1), tolerance of acidosis (*CA9*), cell survival (for example, insulin-like growth factor 1 (*IGF1*)) and metastasis (such as lysyl oxidase (*LOX*))[92]. HIF1α activity may also be influenced by many factors in addition to hypoxia[92], hence targeting HIF1α or its downstream products may additionally kill pseudo-hypoxic tumour cells. Nonetheless, even if not strictly specific to hypoxia, HIF1 inhibitors clearly have considerable potential to suppress resistance to therapy through multiple mechanisms, including the prevention of HIF1-dependent enhancement of endothelial cell radioresistance through cycling hypoxia[94] and blocking of the vasculogenic response to ionizing radiation-induced hypoxia[10].

HIF1α overexpression and its association with poor treatment response and outcome has been demonstrated in an extensive range of human tumours[19,95] (TABLE 2). Multiple components of the HIF1 signalling pathway have been identified as candidate drug targets[96,97] and a wide range of pharmacological approaches have been proposed; surveys of these have been published recently[92,95] (TABLE 4). Several novel agents have undergone Phase I evaluation (such as EZN-2968 (www.ClinicalTrials.gov identifier NCT00466583) and PX-478 (www.ClinicalTrials.gov identifier NCT00522652)), but currently there is no clear clinical evidence of antitumour activity due to HIF1 inhibition. Other agents have been 'repurposed' from their original applications (such as the antibiotic geldanamycin[98]), and have limited specificity for HIF1α. In addition, many new agents have been discovered through phenotypic screens (inhibition of HIF1α signalling) but their direct molecular targets and ability to selectively kill hypoxic cells are not yet well defined. A further interesting strategy for the selective killing of HIF1-expressing cells is the incorporation of a PHD-sensitive oxygen degradation domain (ODD) from HIF1α into cytotoxic proteins, such as a procaspase 3 fusion protein containing both an ODD and a protein transduction domain[99].

The UPR. The elucidation of the role of the UPR in oxygen sensing and hypoxic cell survival has extended the potential molecular targets for drugging hypoxic cells[100]. Oxygen is the preferred terminal electron acceptor in the redox relay required for disulphide bond formation in protein folding[101]. Severe hypoxia leads to increased levels of unfolded proteins in the endoplasmic reticulum (ER), leading to the induction of the UPR (FIG. 4). The UPR is mediated by three signalling pathways: the PERK–eukaryotic translation initiation factor 2A (eIF2A)–activating transcription factor 4 (ATF4) pathway, the inositol-requiring

Pseudo-hypoxia
The induction of molecular responses analogous to those caused by hypoxia but triggered by other conditions.

Table 3 | **Bioreductive prodrugs of DNA-reactive cytotoxins recently or currently in clinical development**

Prodrug	Current clinical status	Company or institution	Chemical class	Mechanism of activation*	Mechanism of cytotoxicity	One-electron reductases	Two-electron reductases	K_{O2} (μM)
Tirapazamine (SR 4233)	Phase III, cervix (closed)	SRI International/ NCI	Aromatic *N*-oxide	1, 3 [R•]	Complex DNA damage	CYPOR, iNOS	NQO1‡	~1
Apaziquone (E09)	Phase III, bladder (closed)	Spectrum	Quinone	1, 4 [X,Y]	ICL	CYPOR	NQO1	
TH-302	Phase I/II, multiple (active)	Threshold	Nitro	1, 3 [D]	ICL	CYPOR		~10§
PR-104	Phase I/II, leukaemia (active)	Proacta and University of Auckland	Nitro	1/2, 4, 5, 6 [Y,Z]	ICL	CYPOR, iNOS, MTRR, NDOR1	AKR1C3	~0.1
Banoxantrone (AQ4N)	Recent Phase I/II	Novacea	Aliphatic *N*-oxide	2, 5 [Y]	TOPOII	iNOS	CYP3A4, CYP2S1	
Caricotamide (EP-0152R) plus tretazicar (CB1954)	Phase II, HCC (discontinued)	BTG	Nitro	1 /2, 4, 5, 6 [Y,Z]	ICL	CYPOR, iNOS	NQO1, NQO2	
RH1	Recent Phase I	CRUK	Quinone	1, 4 [X,Y]	ICL		NQO1, NQO2	
NLCQ-1	Preclinical	Evanston Hospital	Nitro	1, 4, 5	TOPOII or multiple?	CYPOR		~1§
SN30000 (CEN-209)	Preclinical	Centella and University of Auckland	Aromatic *N*-oxide	1, 3 [R•]	Complex DNA damage	CYPOR		~1
SN29730	Preclinical	University of Auckland	Nitro	1, 4, 5, 6 [Z]	Adenine *N*3 alkylation	CYPOR		
KS119W	Preclinical	Yale University	Nitro	1, 4, 5, 6 [D]	Guanine *O6* ICL	B5R, CYPOR		

See FIG. 3 for chemical structures. AKR1C3, aldo-keto reductase 1C3; B5R, NADH-cytochrome *b5* reductase, CRUK, Cancer Research UK; CYP, cytochrome P450; CYPOR, NADPH–cytochrome P450 reductase; HCC, hepatocellular carcinoma; ICL, DNA interstrand crosslink; iNOS, inducible nitric oxide synthase; MTRR, methionine synthase reductase; NCI, US National Cancer Institute; NDOR1, NADPH-dependent diflavin oxidoreductase 1; NQO, NAD(P)H dehydrogenase [quinone]; TOPOII, topoisomerase II. *Reaction numbers refer to FIG. 2A. Active cytotoxins (X,Y etc in FIG. 2A) are shown in square brackets. ‡Detoxifying. §Gas phase O_2 concentration[66] (K_{02} values of 2-nitroimidazoles are typically much lower based on solution oxygen concentrations). See also Supplementary information S1 (tables) for tables with references.

enzyme 1 (IRE1; also known as ERN1)–X-box binding protein 1 (XBP1) pathway and the ATF6 pathway. These pathways activate responses to suppress protein synthesis, stimulate protein degradation in the ER, and activate apoptosis and autophagy to resolve ER stress[93]. An additional mechanism of activation of UPR by hypoxia is the stabilization of ATF4 through loss of its oxygen-dependent PHD3-mediated degradation[102]. Gene knockout and RNA interference studies have demonstrated that the PERK–eIF2A–ATF4 and IRE1–XBP1 pathways contribute to hypoxic cell survival[102–104].

Two drug strategies are being pursued to kill hypoxic cells selectively through UPR targets (TABLE 4). One approach seeks to inhibit the UPR by targeting PERK, ATF4 and IRE1. High-throughput screens and *in vivo* luminescence-based assays for UPR inhibitors have been reported[105], as have first-generation inhibitors of the endonuclease domain of IRE1 (REFS 106,107). Further drug discovery will be facilitated by the availability of crystal structures of the endonuclease domain of yeast IRE1 (REF. 108). A second approach seeks to exacerbate ER stress in order to overwhelm the UPR on the assumption that the UPR is near its capacity in hypoxic cells. Evidence that the ER stressors thapsigargin and bortezomib elicit hypoxia-selective cytotoxicity *in vitro* supports this approach[109].

mTOR. As a key node for the integration of the signals regulating cellular energy and nutrient status, mTOR presents a potential target for hypoxic cell killing. Under hypoxia, mTOR complex 1 (mTORC1) kinase activity is restricted through multiple mechanisms (FIG. 4), resulting in the suppression of protein synthesis to an extent that depends on the severity and duration of hypoxia[110]. The mechanisms include activation of the tuberous sclerosis 1 (TSC1)–TSC2 complex through the HIF1 target gene DNA-damage-inducible transcript 4 (*DDIT4*; also known as *REDD1*)[111] and through increased AMP-activated protein kinase (AMPK) activity under hypoxia[110,112]. In addition, hypoxia induces the HIF1 target gene *BNIP3*, which inhibits mTORC1 through RAS homologue enriched in brain (RHEB)[113]. The resulting suppression of mTORC1 has multiple effects on transcription and translation, the latter in part owing to hypophosphorylation of eIF4EBP1, which leads to sequestration of eIF4E and thus inhibition of cap-dependent translation. This results in preferential cap-independent translation of a subset of mRNAs including *HIF1A* and *VEGFA*. Hypoxia has been proposed to have a dual role in tumour cell survival through modulation of mTORC1 (REF. 93). In small, early stage tumours, moderate hypoxia inhibits tumour growth through mTORC1 suppression, providing a selective pressure for abrogation of the pathway. In larger, late stage tumours, mTORC1 suppression by hypoxia may be an

Cap-dependent translation
Translation initiated by binding of the eIF4F complex to the methyl-7-G(5′)pppN structure (cap) at the 5′ end of the mRNA.

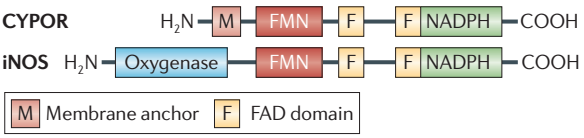
adaptive response in the face of energy limitations, thus favouring hypoxic cell survival. If so, the consequences of further inhibiting mTORC1 in hypoxic cells are difficult to predict. Several studies have explored the activity of mTOR inhibitors in hypoxic cells (TABLE 4). Rapamycin provided hypoxia-selective antiproliferative effects on HT29 cells and, when combined with low dose irinotecan, gave increased hypoxic cell killing *in vitro* and increased tumour control *in vivo*[114]. Treatment with WYE 125132, a potent and specific mTOR kinase inhibitor, gave substantial tumour control in a range of models and blocked HIF1α and HIF2α accumulation under hypoxic conditions, leading to reduced hypoxic adaptation[115].

Targets downstream of the primary hypoxia-sensing pathways. The hypoxia-induced HIF, UPR and mTOR signalling pathways are highly interactive networks that influence many downstream gene products and processes that have potential as therapeutic targets. Here we outline some of the downstream targets under consideration for selective killing of hypoxic cells.

Recent studies have shown that the UPR activates autophagy to ameliorate hypoxic stress[6,116], and that inhibition of autophagy with chloroquine or 3-methyladenine causes selective hypoxic cell killing[6].

Metabolic reprogramming in tumour cells, most famously demonstrated by the shift to aerobic glycolysis (known as the Warburg effect), is in part mediated by HIF1 (REF. 117) and mTOR[7], and is therefore linked to hypoxia. This metabolic switch is also regulated by many other signalling nodes (especially by MYC, p53 and the PI3K–AKT pathway) and reflects the re-gearing of metabolism to support biosynthetic programmes and antioxidant defences to drive tumour cell growth[7,118]. Although the shift from oxidative phosphorylation is not confined to hypoxic cells, the dependence on glycolytic ATP generation creates a vulnerability for these cells because they can no longer call on the residual mitochondrial oxidative phosphorylation, which still contributes significant ATP generation in aerobic tumour cells[119]. This reliance on glycolysis makes hypoxic tumour cells highly sensitive to suppression of glycolytic flux, hence glucose analogues that inhibit glycolysis (TABLE 4) produce striking hypoxia-selective cytotoxicity *in vitro*[120]. The most widely studied compound of this class, 2-deoxy-D-glucose (2DG), is phosphorylated by hexokinases to the corresponding 6-phosphate. This phosphorylated analogue inhibits both hexokinases and phosphoglucose isomerase (GPI), which catalyses the next step in glycolysis[119]. The 2-fluoro analogue of 2DG is a more potent glycolytic inhibitor and hypoxic cytotoxin[121]. 2DG has been evaluated in clinical trials, but the results have not been reported; toxicity to other highly glucose-dependent tissues (such as the brain, retina and testes) represents a potential challenge in the further clinical development of this approach.

Box 1 | **Identity of prodrug-activating one-electron reductases**



Enzymes that catalyse one-electron transfer to prodrugs are central players in hypoxia-selective bioreduction (FIG. 2A). Their identification is an urgent priority to enable profiling of individual tumours, but has proven challenging. The best characterized enzyme is the diflavin reductase NADPH–cytochrome P450 reductase (CYPOR; also known as POR), which catalyses an intramolecular redox shuttle in which a hydride ion (H^-) is transferred from the NADPH domain to the FAD domain, which then transfers electrons to the terminal one-electron donor flavin mononucleotide (FMN) domain (see the figure). CYPOR reduces non-mitochondrial cytochrome P450s (CYPs) and has broad substrate specificity for xenobiotics with one-electron reduction potentials that are similar to or higher than its FMN and FAD redox centres, including many bioreductive prodrugs (TABLE 3).

The nitric oxide synthases (NOSs) have diflavin (FMN and FAD) reductase domains that are homologous to CYPOR, but NOSs reduce an intramolecular haem prosthetic group in the oxygenase domain, which is responsible for nitric oxide synthesis. As for CYPOR, the transferred electron can be intercepted by small molecule electron acceptors such as tirapazamine (TPZ) and quinones[163,164]. Interest has focused on the inducible NOS (iNOS; also known as NOS2) isoform because it is highly expressed in some tumours[165,166] including by macrophages that accumulate in hypoxic zones[167]. Notably, iNOS is upregulated under hypoxia through the binding of hypoxia inducible factor 1 (HIF1) to the transcription factor interferon regulatory factor 1 (IRF1)[168,169]. This leads to localized iNOS expression in hypoxic regions of tumours[170], which provides an additional mechanism of hypoxic selectivity for its substrates. However, given that iNOS expression in tumours is often predominantly stromal[166], this enzyme will be best exploited by bioreductive prodrugs that generate cytotoxic metabolites with an efficient bystander effect. In this regard it is notable that the prodrugs AQ4N[171], CB 1954 (REF. 172) and PR-104A[173] are activated by iNOS under hypoxia; each provides efficient bystander effects and thus has potential for exploiting hypoxic expression of iNOS in the tumour stroma. The tropism of macrophages for hypoxic regions of tumours is also being exploited for the delivery of prodrug-activating enzymes, using adenoviral transduction of *CYPOR* and hypoxia response element (HRE)-regulated *CYP2B6* to activate cyclophosphamide[174]. Increased hypoxic activation of TPZ has previously been demonstrated by transduction of tumour cells with HRE-driven *CYPOR*[175], suggesting the potential for further enhancing hypoxic targeting by bioreductive prodrugs by combining these approaches.

PR-104A can also be activated under hypoxia by the other members of the diflavin reductase family, NADPH-dependent diflavin oxidoreductase 1 (NDOR1) and methionine synthase reductase (MTRR)[173]. Other flavoproteins capable of one-electron prodrug activation include NADH-cytochrome *b*5 reductases[176], ferredoxin reductase (FDXR)[177], xanthine oxidase[55] and xanthine dehydrogenase, which is also capable of two-electron reduction[178]. However, much needs to be learned about the relative activity of these and other reductases in hypoxic regions of human tumours.

There is much interest in inhibiting other targets that can be rate-limiting for glycolysis, and which might offer greater tumour selectivity, including the HIF1-regulated facultative glucose transporter GLUT1, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatases (PFKFBs) and the tumour-specific pyruvate kinase M2 (PKM2) isoform. Elevated GLUT1 levels has been described in a wide range of tumour types and has been demonstrated to be a negative prognostic indicator[122]. Many experimental GLUT1 inhibitors, such as phloretin, have multiple molecular targets or act indirectly, but recent examples (fasentin[123] and

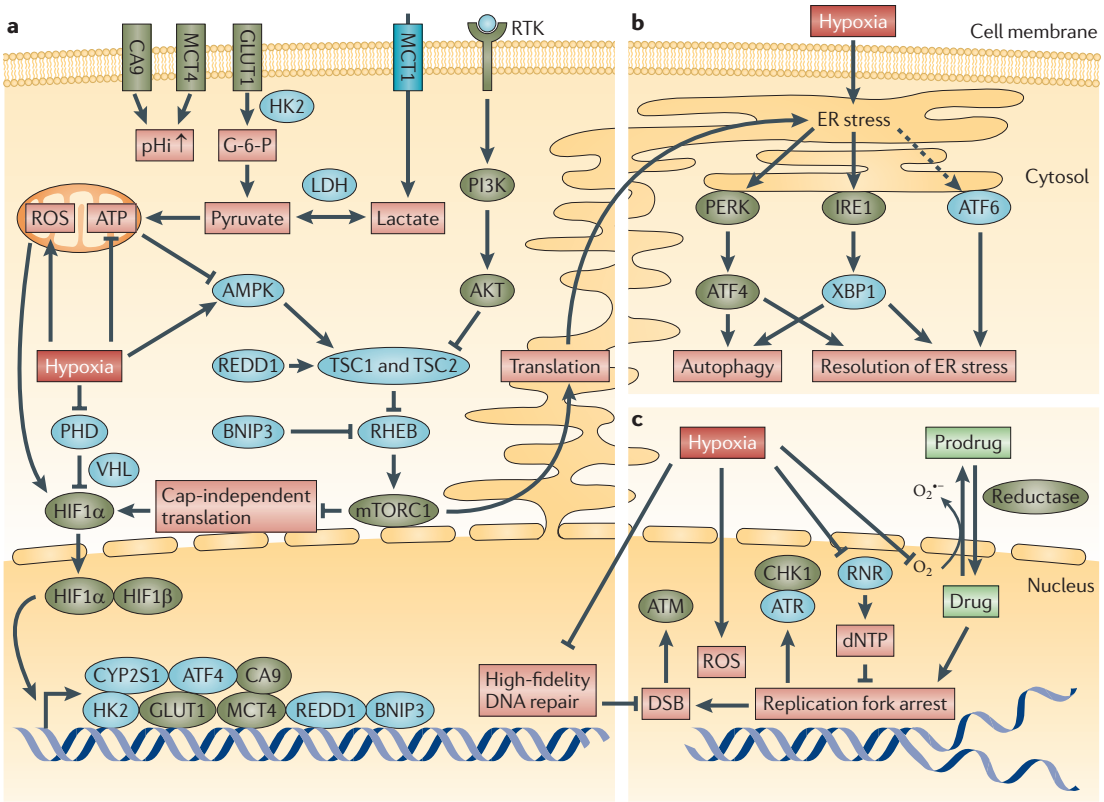


Figure 4 | **Potential molecular targets for killing hypoxic cells in the oxygen-responsive signalling pathways that mediate adaptation to hypoxia. a** | The hypoxia-inducible factor (HIF)–mTOR central metabolism module. Hypoxia inhibits prolyl hydroxylase domain (PHD)-mediated degradation of HIF1α, which allows its dimerization with HIF1β (also known as ARNT) and transcription of a range of genes associated with metabolic reprogramming (including hexokinase 2 (*HK2*) and the glucose transporter GLUT1 (encoded by *SLC2A1*)) and control of intracellular pH (pHi), such as monocarboxylate transporter 4 (*MCT4*) and carbonic anhydrase 9 (*CA9*). Also, the ability of aerobic tumour cells to use lactate in place of glucose for oxidative phosphorylation has been suggested to allow glucose to diffuse to hypoxic cells, which are highly glucose-dependent, defining the lactate transporter MCT1 as a potential target (potential target proteins are shown in green). Hypoxia induces the formation of reactive oxygen species (ROS), which stabilize HIF1α. Hypoxia also inhibits mTOR complex 1 (mTORC1) through the HIF1-dependent transcription of DNA damage-inducible transcript 4 (*DDIT4*, which encodes REDD1) and *BNIP3* and through AMP-activated protein kinase (AMPK) signalling. This inhibition results in the hypophosphorylation of eukaryotic translation initiation factor 4E-binding protein 1 (eIF4EBP1), which favours cap-independent translation of a subset of transcripts, including *HIF1A* and provides an mTOR–HIF1 regulatory loop. Receptor tyrosine kinases (RTKs) also modulate HIF1α translation through mTOR and other pathways in some cell lines and can also influence hypoxic survival responses. **b** | The unfolded protein response (UPR) module. Hypoxia, through the lack of oxygen to act as the ultimate electron acceptor in disulphide bond formation, impairs protein folding in the endoplasmic reticulum (ER). This leads to activation of the UPR, through PRKR-like endoplasmic reticulum kinase (PERK; also known as eIF2AK3), inositol-requiring enzyme 1 (IRE1; also known as ERN1) and potentially activating transcription factor 6 (ATF6), which supports hypoxic cell survival. **c** | DNA damage response module. Severe hypoxia inhibits ribonucleotide reductase (RNR), leading to replication fork arrest and protective ataxia telangiectasia and Rad3-related (ATR) signalling. Production of ROS in hypoxic cells, and especially on re-oxygenation, leads to DNA double-strand breaks (DSBs), which activate ataxia telangiectasia mutated (ATM) signalling. Thus, DNA damage signalling pathways provide potential targets for hypoxia-selective cell killing. Hypoxia also reduces high fidelity DNA repair (by, for example, homologous recombination (HR), which leads to sensitivity to poly(ADP-ribose) polymerase (PARP) inhibitors). In addition, hypoxia permits activation of bioreductive prodrugs, mainly by preventing redox cycling of the prodrug radical anions generated by one-electron reductases. The resulting cytotoxic drugs typically induce DNA replication fork damage, exacerbated by suppression of HR in hypoxic cells, leading to cell death. CYP2S1, cytochrome P450 2S1; G-6-P, glucose-6-phosphate; LDH, lactate dehydrogenase; RHEB, RAS homologue enriched in brain; TSC, tuberous sclerosis; VHL, von Hippel-Lindau tumour suppressor; XBP1, X-box binding protein 1.

Table 4 | **Representative examples of pharmacological approaches to molecular targets in hypoxic cells***

Pathway	Target	Agent	Class
HIF1α expression	HIF antisense mRNA	EZN-2968	RNA oligonucleotide
	Topoisomerase I	Topotecan	Camptothecin analogues
	Multiple	PX-478	Melphalan *N*-oxide
	Translation	Digoxin	Cardiac glycoside
	HSP90	Geldanamycin and tanespimycin (17-AAG)	Benzoquinone ansamycin antibiotics
HIF1 transcription	HIF–p300 binding	Chetomin and analogues	Dithiodiketopiperazine
	Thioredoxin 1	PX12	Imidazole disulphide
		PMX290	Indoloquinol
	DNA binding	Echinomycin	DNA intercalator
HIF1 target gene products	CA9 and CA12	Aryl sulphonamides	Sulphonamide zinc binders
	GLUT1	Glufosfamide	Glucose isophosphoramide mustard
		2-GLU-SNAP	Glucose SNAP conjugate
		Fasentin	Oxobutanilide
		STF-31154	Unknown
	HK2	5TDG, 2DG, 2FDG	Glycolysis inhibitors
	MCT1	α-cyano-4-hydroxycinnamate	Lactate transport inhibitor
Receptor tyrosine kinases	VEGFR	Bevacizumab	Monoclonal antibody
	EGFR	Gefitinib and erlotinib	ATP competitive kinase inhibitors
		Cetuximab	Monoclonal antibody
RAS–MAPK signalling	BRAF	Sorafenib	ATP competitive kinase inhibitor
mTOR	mTORC1	Rapamycin and everolimus	Allosteric binders of FKBP12-rapamycin binding domain
		WYE-125132	ATP-competitive mTOR kinase inhibitor
	Autophagy	Chloroquine	Lysosomal pH
UPR	HSP90	Geldanamycin and 17-AAG	Benzoquinone ansamycin antibiotic
	IRE1	Salicaldehydes	IRE1 inhibitor
	26S proteasome	Bortezomib	Boronic acid tripeptide
		Nelfinavir and ritonavir	HIV protease inhibitors
	SERCA	2,5-Dimethyl celecoxib	Celecoxib analogue

CA, carbonic anhydrase; DG, deoxy-D-glucose; EGFR, epidermal growth factor receptor; FDG, fluorodeoxyglucose; FKBP12, FK506 binding protein 12; GLUT1, glucose transporter 1; HIF, hypoxia-inducible factor; HK2, hexokinase 2; HSP90, heat shock protein 90; IRE1, inositol-requiring enzyme 1 (also known as ERN1); MCT1, monocarboxylate transporter 1; mTORC1, mTOR complex 1; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; SNAP, S-nitroso-acetyl-penicillamine; UPR; unfolded protein response; VEGFR, vascular endothelial growth factor receptor. *See also Supplementary information S1 (tables) for tables with references.

STF-31154 (REF. 124)) target GLUT1 directly. The shift to glycolysis is accompanied by increased generation of pyruvate and its conversion to lactate by lactate dehydrogenase A (LDHA). The lactate transporter monocarboxylate transporter 1 (MCT1) has been suggested as a target for killing hypoxic cells by glucose starvation, through a novel mechanism of metabolic symbiosis[125]. This study showed that aerobic tumour cells expressing MCT1 can use lactate as a preferred substrate for respiration, and further demonstrated that inhibition of MCT1 by α-cyano-4-hydroxycinnamate increases glucose consumption *in vitro* and tumour radiosensitivity[125]. The proposed model is that the stimulation of glucose consumption in aerobic tumour cells compromises glucose penetration into hypoxic regions, leading to the selective death of hypoxic cells in tumours. However, laboratory tools such as α-cyano-4-hydroxycinnamate are not particularly selective for the MCTs[126] and one class of selective MCT1 inhibitors has been identified as an immunomodulator[127], raising concerns about the selectivity of such an approach for targeting hypoxic cells.

One of the consequences of the glycolytic shift, driven in part by hypoxia, is that increased generation of metabolic acids further compromises hypoxic cell survival. Disruption of pH homeostasis by targeting MCTs (such as MCT1 and MCT4) and carbonic anhydrases in hypoxic tumour cells has been proposed as a tumour-selective approach[128]. MCT4 is upregulated in a HIF1α-dependent manner[129] and increased expression of MCT4 in tumour cells has been demonstrated[130]. MCT4 export of lactate and H^+ prevents intracellular acidification and assists in the remodelling of the extracellular milieu, but specific inhibitors of MCT4 have yet to be reported.

Carbonic anhydrases are metalloenzymes that catalyse the reversible hydration of carbon dioxide to carbonic acid. The expression of CA9 and CA12 is

controlled by HIF1 (REF. 131) and CA9 is also regulated through the UPR by ATF4 (REF. 132). Despite generating H^+ and HCO_3^- with equivalent stoichiometry at the extracellular catalytic domain of these transmembrane proteins, linked bicarbonate transporters raise the intracellular pH to protect hypoxic cells[128]. Silencing both CA9 and CA12 resulted in marked inhibition of the growth of LS174 human colon carcinoma cell xenograft tumours[131]. Extensive drug development efforts have identified a range of compounds with varying selectivity for CA9 and CA12; several compounds inhibited tumour growth and metastasis selectively in CA9-positive tumour models[133].

Molecular targets in DNA damage response and repair pathways. Inhibitors of DNA damage signalling and DNA repair have the potential to exploit changes in these pathways in hypoxic cells[134–136]. Three approaches have recently been considered. The first is to exploit activation of the DNA damage response in hypoxic cells. Severe hypoxia rapidly induces replication arrest through a HIF1- and p53-independent mechanism[137]. Recent evidence indicates this is due to depletion of dCTP, dGTP and dATP pools[138], reflecting the requirement of class 1a (eukaryotic) ribonucleotide reductases for molecular oxygen[139]. Single-stranded DNA at stalled replication forks then induces ataxia telangiectasia and Rad3-related (ATR)–CHK1 signalling, which is required to maintain replication fork integrity. Consistent with this, knockdown of CHK1 is selectively toxic to hypoxic cells[140]. This ATR-mediated replication arrest is reversible if cells are re-oxygenated within a few hours, but re-oxygenation then induces ROS-mediated DNA damage, including double-strand breaks that activate the kinase ataxia-telangiectasia mutated (ATM)[141], potentially providing sensitivity to inhibitors of ATM signalling.

A second strategy is to exploit defects in DNA repair in hypoxic cells. ATR- and ATM-mediated signalling in hypoxic cells can help to facilitate DNA repair. For example, hypoxia stimulates CHK2-mediated Ser988 phosphorylation of BRCA1[142], which stimulates its activity in HR. However, hypoxia also downregulates expression of key HR proteins such as RAD51 and BRCA1 through HIF1-independent repression of transcription and translation[136]. In addition, hypoxia suppresses RAD51 expression in breast cancer initiating cells through HIF1-dependent upregulation of the Polycomb protein enhancer of zeste homologue 2 (EZH2)[143]; *RAD51* mRNA has also recently been shown to be downregulated in hypoxic regions of 9L gliomas by laser-capture microdissection of etanidazole pentafluoride (EF5)-stained tissue[144]. Hypoxia-mediated suppression of HR in chronically hypoxic cells[145,146] confers an increased sensitivity to DNA-damaging cytotoxins[146], which may make a significant contribution to the activity of bioreductive prodrugs that deliver such cytotoxins to hypoxic cells. Notably, hypoxia-induced downregulation of HR creates the same phenotype that sensitizes BRCA1 or BRCA2 homozygous mutant cells to PARP1 inhibition. Recently a synthetic lethal interaction has been demonstrated for hypoxia and genetic deletion or chemical inhibition of PARP1, analogous to that for BRCA1 or BRCA2 mutations, and the PARP1 inhibitor veliparib (also known as ABT-888) has been shown to selectively reduce the proportion of radioresistant (that is, hypoxic) cells in RKO colon carcinoma xenografts[147]. The authors point to the potential for synthetic lethal interactions between hypoxia and inhibitors of other repair pathways downregulated by hypoxia.

A third strategy is to pharmacologically reactivate p53 to restore hypoxia-mediated apoptosis[135]. Small molecules that are in development for p53 reactivation include APR-246 (also known as PRIMA-1), which restores transcriptional activity of mutant p53, and Nutlin-3 and RITA, which interfere with MDM2-mediated p53 degradation[148]. RITA also induced a DNA damage response that appears to contribute to its stimulation of p53-dependent apoptosis, but cell killing was similar in hypoxic and aerobic cells[149].

Hypoxia and personalized cancer medicine

As in other aspects of cancer medicine, emerging technologies for profiling individual tumours have the potential to revolutionize the development of hypoxia-targeted agents. Indeed, the heterogeneity in tumour hypoxia at the broader human population level, even within a single disease subtype, means that successful development of hypoxia-targeted agents is probably a forlorn hope unless hypoxic tumours can be identified prospectively. Studies with advanced head and neck squamous cell carcinomas (HNSCCs), in which hypoxia has been demonstrated to be a negative prognostic factor using every type of diagnostic tool available (TABLE 2), are instructive in this regard. A large, relatively homogenous (stage T2–T4 laryngeal) series of HNSCC samples showed evidence of hypoxia by both pimonidazole and CA9 immunostaining in the majority of tumours, but with extreme variability[150]. The need to quantify (not just to detect) hypoxia is illustrated by a meta-analysis of oxygen-electrode studies, which suggested that hypoxia compromised overall survival in patients with advanced HNSCC undergoing chemoradiation treatment but only in the subset of patients with the most extensive hypoxia[151]. This situation is different from the subcutaneous xenograft models widely used in preclinical studies, in which essentially all tumours display extensive hypoxia; these models thus tend to over-represent the target (and will over-predict activity) relative to autochthonous tumours in humans.

Thus there is currently much interest in the further development of hypoxia diagnostics as predictive biomarkers[18,19,152,153]. Although studies using invasive methods (TABLE 2) have been important in establishing the significance of tumour hypoxia at the population level, broader clinical application for stratifying patients will require less-invasive tools such as positron emission tomography (PET) imaging (BOX 2). There is also great potential for minimally invasive serum-based diagnostics and global gene expression signatures for the identification of hypoxia (TABLE 2).

Synthetic lethal interaction
In genetics, an interaction between two non-lethal mutations that, in combination, confer lethality. In chemical genetics, this term can refer to interaction between a drug and mutation that confers greater drug-sensitivity than with the wild type.

Autochthonous tumours
Tumours that arise in the host being studied, as distinct from tumours introduced by transplantation.

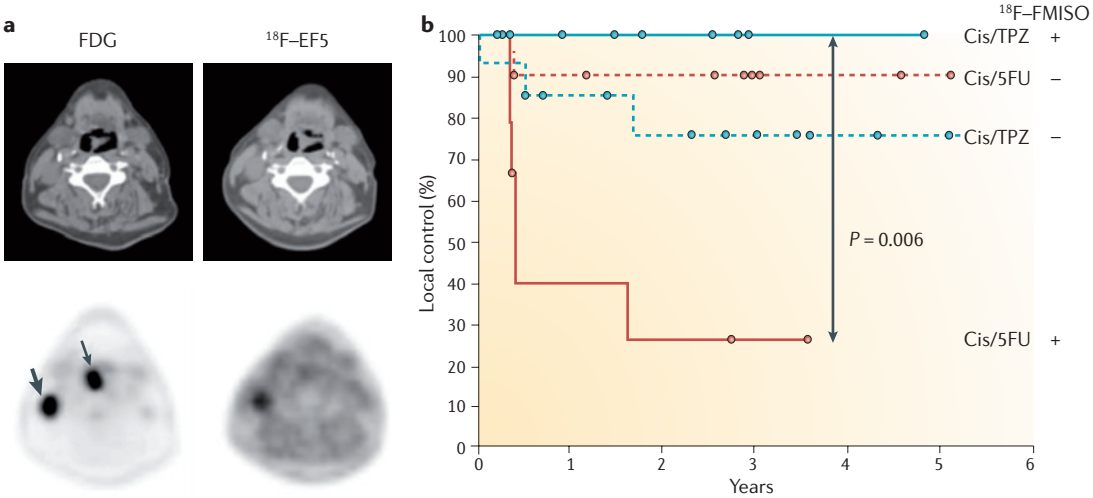
The presence of hypoxia is a necessary but not sufficient condition for hypoxia-targeting, given that there are other crucially important determinants of sensitivity to such agents. For bioreductive prodrugs, the molecular targets are in effect the specific reductases in hypoxic cells for which these compounds are substrates. Although identification of these enzymes is incomplete (BOX 1), their activity clearly varies widely between tumours. The need for reductase profiling to identify tumours potentially responsive to bioreductive prodrugs has long been recognized[154], but only now are the tools becoming available to address this requirement. In addition, there is a further set of molecular targets, for the active drug metabolites, which brings into play many potential mechanisms of drug resistance. Given that most bioreductive prodrugs generate DNA damage that is repaired by HR, the validation of biomarkers for this repair pathway (currently driven by predicting the sensitivity to PARP inhibitors and cytotoxic chemotherapy[155–157]) has strong potential to affect their development.

Clearly, the diagnostic tools for selecting patients for treatment with hypoxia-targeted drugs need to be matched to the specific therapeutic agent. Thus, one would expect the preferred diagnostic for a bioreductive prodrug to be an exogenous probe that is activated through bioreductive metabolism (by similar enzymes and with similar oxygen-dependence to the therapeutic agent). As an example, binding of the 2-nitroimidazole probe EF5 reports activity of the one-electron reductases that activate SN30000, as well as reporting hypoxia, making it a potential dual probe for both of these stratification biomarkers[158]. By contrast, endogenous markers of hypoxia-responsive signalling pathways will be more appropriate for agents that target such pathways. It is noteworthy that there tends to be poor correlation between different hypoxia markers in both

Box 2 | **PET imaging for tumour hypoxia**

The variability in levels of hypoxia among individual tumours, even within a single disease subtype, calls for tools that can be used to quantify tumour hypoxia in a clinical setting. Positron emission tomography (PET) methods are undergoing active development in this context[152]. One strategy depends on radiolabelled antibodies against carbonic anhydrase 9 (CA9)[179,180], which would be of value for the selection of patients for treatment with CA9-targeted therapeutics[133]. To the extent that CA9 can be considered a specific hypoxia-inducible factor 1 (HIF1) reporter[132,181], and that HIF1 activity is regulated by hypoxia[92], this approach also has potential for monitoring hypoxia.

The most widely studied PET strategy depends on entrapment of 2-nitroimidazole probes — such as fluoromisonidazole (FMISO), fluoroazomycinarabinofuranoside (FAZA) and etanidazole pentafluoride (EF5) — in hypoxic cells as a result of their bioreductive metabolism[152]. The mechanism is analogous to that for one-electron (oxygen-inhibited) metabolic activation of bioreductive prodrugs, subsequently generating nitroso and hydroxylamine metabolites (X and Y in FIG. 2A), which react covalently with intracellular thiols. The resulting protein adducts can be detected by immunohistochemistry (FIG. 1a), which requires a tumour biopsy, but ^{18}F-labelled versions of the same compounds have been adapted for non-invasive PET imaging. The PET–computerized tomography (CT) scan shown in part **a** of the figure demonstrates a difference in ^{18}F–EF5 entrapment in two lesions in the same patient that both rapidly metabolize ^{18}F–fluorodeoxyglucose (FDG), suggesting that the lesion marked with the wide arrow is more hypoxic than that marked with the thin arrow. The related 2-nitroimidazole probe ^{18}F–FMISO has been used to evaluate hypoxia in a small subset of patients in clinical trials of the bioreductive prodrug tirapazamine (TPZ) combined with cisplatin (cis) and radiotherapy, versus 5-fluorouracil (5FU) combined with cisplatin and radiotherapy for advanced head and neck squamous cell carcinoma (HNSCC). As shown in part **b** of the figure, a retrospective analysis demonstrated a marked advantage of the TPZ-containing regimen compared to the 5FU-containing regimen in patients with hypoxic tumours (solid lines, ^{18}F–FMISO-negative)[183]. This notable result points the way for future trials of hypoxia-targeted agents, but, regrettably, stratification for hypoxia was not used in subsequent unsuccessful Phase III trials of TPZ in this same setting[184]. Part **a** of the figure is reproduced, with permission, from REF. 182 © (2008) Society of Nuclear Medicine, Inc. Part **b** of the figure is modified, with permission, from REF. 183 © (2006) The American Society of Clinical Oncology.



Network medicine
Analysis of biological networks to derive understanding of disease and therapy.

preclinical and clinical studies[150,159]. Ultimately, paired diagnostics and therapeutics will need to be validated in prospective clinical trials, despite the logistical and regulatory challenges that this presents.

Conclusions and perspective

This Review has considered the two main approaches to the selective killing of hypoxic cells in tumours, with different strengths and weaknesses. Bioreductive prodrugs achieve striking selectivity between aerobic and severely hypoxic cells in culture, typically with potency differentials in the order 10–1,000-fold. By contrast, inhibition of molecular targets in hypoxic cells typically gives much more modest cytotoxicity differentials. However, these targeted inhibitors offer a more benign toxicity profile, which is distinctly different from that of cytotoxic therapy, and therefore have greater opportunity for combination with current standards of care. Compatibility with existing therapy is fundamentally important for the clinical translation of these targeted drugs, given that hypoxic cells represent only a minority subpopulation in most tumours (although a critically important one). Therefore, monotherapy activity is not a realistic expectation for hypoxia-selective agents that are strictly on-mechanism unless exceptional requirements can be met, such as a very long residence time in tumours (to exploit fluctuating hypoxia) or efficient, long-range bystander killing. Bioreductive prodrugs that generate molecularly targeted drugs as effectors, rather than DNA-damaging cytotoxins, arguably offer an opportunity to combine the best features of both classes of drug (high hypoxic selectivity and more benign toxicity), but are at an early stage of development.

Although much has already been learned about the molecular responses to hypoxia, the identification of the most useful molecular targets in hypoxic cells is far from complete. While new targets with roles in hypoxic cell survival continue to be identified, the highly interactive nature of the PHD–HIF, mTOR, UPR–autophagy and DNA damage response modules (FIG. 4) makes it difficult to identify the vulnerabilities of hypoxic cells that can best be exploited as drug targets. The results of unbiased whole-genome screens, analogous to the RNA interference screens used to identify synthetic lethal interactions with chemotherapy[160], are eagerly awaited. Ideally, these screens will compare multiple cancer cell lines with normal cells, under hypoxia, to reveal targets that provide selectivity for hypoxia in the context of cancer genomes, and will be interpreted in a network medicine framework[161]. A better definition of the preferred molecular targets will make it feasible to design small molecules of greater specificity, and to move beyond the repurposing of drugs that have been developed for other applications, an approach that currently characterizes this field (TABLE 4). In a similar fashion, improved understanding of the human reductases that activate prodrugs will provide opportunities for structure-based design to improve specificity for enzymes that confer tumour selectivity.

Many of the challenges in targeting hypoxic cells are similar for both bioreductive prodrugs and molecularly targeted inhibitors; both need to be designed to address the stringent micropharmacokinetic requirements for efficient penetration to cells distant from blood vessels. This critical issue is still rarely addressed explicitly. Both classes of drugs also need to address, and where possible exploit, off-target effects (such as the aerobic reduction of bioreductive drugs, and the inhibition of hypoxia-independent HIF1 responses to ionizing radiation[162]). An associated challenge is the potential toxicity resulting from physiological hypoxia in normal tissues; there is still little understanding of the contribution of such hypoxia to the dose-limited toxicities of bioreductive prodrugs. In addition, clinical development of all hypoxia-targeted agents suffers from a lack of information about the clinical settings in which hypoxic cells contribute to treatment failure. The notable exception is in chemoradiation treatment of HNSCC, for which there is overwhelming evidence from multiple hypoxic biomarkers that hypoxia compromises outcome (TABLE 3). An additional challenge is the lack of a drug-development culture in the field of radiation oncology, which is the setting in which the impact of hypoxia is most clearly understood.

Perhaps the most crucial requirement for hypoxia-targeting strategies is the development of improved predictive tools for patient stratification. These tools need to evaluate not only hypoxia, but also many other determinants of sensitivity, as discussed above. Ultimately, tumour and host genomic analyses will revolutionize the matching of hypoxia-targeted therapeutics to individual patients. However, extracting information on physiological features such as the severity of hypoxia from genomic data will be challenging, so functional assays such as PET imaging are likely to play a major part in the foreseeable future. Together, this individualized phenotyping has the potential to identify clinical niches for the diverse types of cytotoxins that are already identified as hypoxia-selective, and provide a rational basis for their clinical development.

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Competing interests statement
The authors declare competing financial interests: see Web version for details.

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