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# Hypoxia-activated prodrugs: paths forward in the era of personalised medicine

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Tumour hypoxia has been pursued as a cancer drug target for over 30 years, most notably using bioreductive (hypoxia-activated) prodrugs that target antineoplastic agents to low-oxygen tumour compartments. Despite compelling evidence linking hypoxia with treatment resistance and adverse prognosis, a number of such prodrugs have recently failed to demonstrate efficacy in pivotal clinical trials; an outcome that demands reflection on the discovery and development of these compounds. In this review, we discuss a clear disconnect between the pathobiology of tumour hypoxia, the pharmacology of hypoxia-activated prodrugs and the manner in which they have been taken into clinical development. Hypoxia-activated prodrugs have been evaluated in the manner of broad-spectrum cytotoxic agents, yet a growing body of evidence suggests that their activity is likely to be dependent on the coincidence of tumour hypoxia, expression of specific prodrug-activating reductases and intrinsic sensitivity of malignant clones to the cytotoxic effector. Hypoxia itself is highly variable between and within individual tumours and is not treatment-limiting in all cancer subtypes. Defining predictive biomarkers for hypoxia-activated prodrugs and overcoming the technical challenges of assaying them in clinical settings will be essential to deploying these agents in the era of personalised cancer medicine.

Initial evidence for the existence of hypoxia within tumours was contemporaneous with the discovery of the structure of DNA and preceded the discovery of cellular oncogenes by two decades. In the subsequent years, the prevalence of hypoxia and its association with and contribution to poor prognosis (Brizel *et al*, 1996; Hockel *et al*, 1996) and treatment failure (Nordsmark *et al*, 2005) in multiple indications provided a compelling rationale for clinical-stage drug development programmes targeting or exploiting this feature of the tumour microenvironment. The majority of these studies have explored prodrugs of antineoplastic agents that are activated in cells by oxygen-inhibited enzymatic reduction (hypoxia-activated prodrugs; HAPs) (Wilson and Hay, 2011; Phillips, 2016). The clinical development of six of these compounds (tirapazamine, apaziquone, banoxantrone, porfiromycin, PR-104 and RH1) has been discontinued, whereas two remain active (evofosfamide/TH-302 and tarloxotinib bromide/TH-4000). However, it was recently reported that evofosfamide did not achieve

primary overall survival endpoints in combination with chemotherapy in two large phase 3 studies in advanced unresectable or metastatic pancreatic adenocarcinoma (MAESTRO; NCT01746979) and in soft tissue sarcoma (TH-CR-406/SARC021; NCT01440088), whereas a phase 2 trial in NSCLC (NCT02093962) was discontinued for futility.

The conspicuous failure of two major registrational programs—those of tirapazamine and evofosfamide—calls for a considered reflection on the rationale for drugging hypoxia, molecule-specific liabilities that may be corrected in subsequent drug discovery efforts, and the manner in which these agents are taken into development. Despite its antiquity, HAP development has yet to fully leverage the advancements in molecular profiling that have enabled targeted treatment of defined tumour subtypes in other areas of medical oncology (Chapman *et al*, 2011; Mateo *et al*, 2015). Indeed, the task of translating these compounds into the clinic has been confounded by the complex biology and

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heterogeneity of the target and failure to clearly define and access—using diagnostic assays fit for commercial drug development—clinical indications in which hypoxia is present or treatment-limiting. The latter is particularly imperative in the context of the HAPs of DNA-reactive cytotoxins, the toxicity profiles of which have frequently required dose reductions in combination with standard of care (Rischin *et al*, 2001; McKeage *et al*, 2012). Of equal importance is the need to develop diagnostics for predicting the sensitivity of individual tumours to HAPs; an effort that may be informed by an increasing understanding of the molecular pharmacology of these agents (Figure 1). In this sense, the challenges for HAPs are reflective of wider trends in the development of precision medicine. In this review, we outline recent developments in the study of tumour hypoxia and the mechanism of action of HAPs and how these may inform the clinical deployment of these prodrugs in the context of personalised cancer medicine.

### HYPOXIA AND OUTCOME: FINDING THE POINT OF MAXIMAL IMPACT

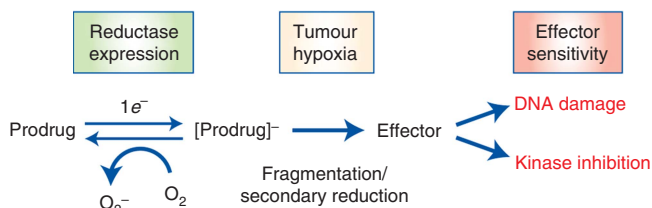
The initiating premise for HAP development was the identification of hypoxia in many types of tumours and its association with treatment resistance and poor prognosis. The strongest such evidence comes from clinical trials incorporating oxygen needle electrode-based measurements in accessible tumours of the head and neck (Nordmark *et al*, 2005), cervix (Fyles *et al*, 1998, 2002), and prostate (Milosevic *et al*, 2012). Similar corroborating evidence in these and many other tumour types has been obtained using indirect assessments of hypoxia. These include exogenous nitroimidazole-based hypoxia probes used in either PET imaging (e.g. [<sup>18</sup>F]-FAZA, [<sup>18</sup>F]-MISO, [<sup>18</sup>F]-HX4) or immunohistochemistry assays (EF5, pimonidazole). Indirect assessments of hypoxia have also been conducted using mRNA or protein-based expression incorporating single or multiple genes (signatures) induced by hypoxia. Based on all such assessments, the levels of hypoxia in virtually every cancer type examined have demonstrated a remarkable diversity across patients. This variation is important, in part because it provides a tool to differentiate patients with distinct clinical prognoses. For example, high levels of hypoxia are

associated with local-regional failure of patients with head and neck squamous cell carcinoma (HNSCC) in a meta-analysis of 397 patients treated with definitive radiotherapy (Nordmark *et al*, 2005). Similarly, high levels of hypoxia predict for worse overall survival in cervix (Fyles *et al*, 1998) and prostate cancer (Milosevic *et al*, 2012) treated with radiotherapy. Levels of hypoxia vary from near zero to almost 100%; however, no clinical factors (e.g. size, stage) or genomic features have been identified that are predictive of these values.

Despite these clinical observations, none of the large HAP clinical registration trials have incorporated biomarkers to identify patients expected to benefit from such therapies. Thus, one explanation for the lack of success of these trials has been the inclusion of patients that have little chance to benefit simply because their tumours have low levels of hypoxia. There are some retrospective studies that support this conclusion. Rischin *et al* (2006) used [<sup>18</sup>F]-MISO PET to assess hypoxia in a sub-study of 45 patients within a large randomised phase 2 study comparing tirapazamine plus chemoradiation to chemoradiation alone for HNSCC. They demonstrated a significant benefit of tirapazamine in patients containing hypoxic tumours, with only one of 19 patients experiencing a local-regional failure, compared to 8 of 13 in the control arm. Data from Toustrup *et al* (2012b) have similarly demonstrated that biomarkers of hypoxia can identify subsets of hypoxic HNSCC that benefit from treatments targeting hypoxic cells. A retrospective study using a 15-gene hypoxia signature (Toustrup *et al*, 2011) to classify patients into ‘more hypoxic’ and ‘less hypoxic’ subsets, demonstrated that the benefit of nimorazole (a hypoxic radiosensitiser) in the Danish Head And Neck Cancer 5 trial (DAHANCA 5) was observed only within the hypoxic group (Toustrup *et al*, 2012b). Five-year local-regional control rates improved from 18 to 49% with nimorazole in the hypoxic group of 114 patients, whereas there was no benefit in the less hypoxic group of 209 patients.

Compared with head and neck cancer, it is more difficult to estimate the proportion of patients with hypoxic tumours expected to benefit from evofosfamide in the recent phase 3 trials in pancreatic adenocarcinoma and soft tissue carcinoma. Intraoperative oxygen needle electrode measurements were made in seven pancreas carcinoma patients and showed high levels of hypoxia (Koong *et al*, 2000). However, a more recent study in 10 patients using pimonidazole showed that like other cancers, hypoxic levels show a wide range of values from 0–26% (Dhani *et al*, 2015). This is particularly relevant, given that pimonidazole activation and binding occurs through a similar oxygen-dependent enzymatic reaction as evofosfamide (described below). Although completion of larger ongoing trials in pancreatic cancer using pimonidazole and/or [<sup>18</sup>F]-FAZA PET will be needed to fully understand the variation in hypoxia, it is highly likely that many patients in the MAESTRO trial will have had levels of hypoxia insufficient to benefit from evofosfamide.

In addition to the need for hypoxia, there are other important considerations relevant to the potential efficacy of HAPs in different disease contexts. It has become clear that hypoxia, even when present, is not equally important in all patients. This is particularly relevant in curative settings where HAPs are expected to be beneficial only when hypoxic cells are actually limiting therapy outcome. As described above, this has been shown definitively in HNSCC treated by radiotherapy. However, even in this case, the importance of hypoxia is not equivalent in all patients. HNSCC consist of multiple disease subtypes, including those caused by infection with human papillomavirus (HPV). HPV infection is associated with significantly improved overall survival and the aetiology of this disease, including the underlying causal genetic mutations, is distinct from HPV-negative disease (Ang *et al*, 2010; TCGA Network, 2015). Interestingly, the range and levels of hypoxia, as assessed by the 15-gene signature described



**Figure 1. Molecular pharmacology of HAPs.** Aromatic nitro (e.g. evofosfamide, tarloxotinib, PR-104, nitroCBI) and N-oxide (e.g. tirapazamine, SN30000) HAPs are activated in tumours by a process that is initiated by enzymatic one-electron reduction to yield a prodrug radical anion. In well-oxygenated tissue, the unpaired electron is rapidly scavenged from this reducing radical by molecular oxygen, resulting in a futile redox cycle. In the absence of oxygen, the radical anion either fragments or is reduced further to produce a cytotoxic effector species that engages a pharmacological target (e.g. by kinase inhibition or damaging DNA through alkylation, oxidation or poisoning of topoisomerase II). Collectively, this understanding of the molecular pharmacology of HAPs implies that presence of tumour hypoxia, expression of one-electron reductases in addition to cell-intrinsic modifiers of sensitivity of the cytotoxic effector may influence the efficacy of HAPs in individual malignancies.

above, appear similar in HPV-positive and HPV-negative HNSCC (Toustrup *et al*, 2012a, b). However, a retrospective analysis of the DAHANCA 5 study showed that nimorazole benefitted patients with hypoxic HPV-negative tumours but had no benefit in HPV-positive HNSCC (Lassen *et al*, 2010). Trinkaus *et al* (2014) similarly demonstrated that the addition of tirapazamine to chemoradiation for HNSCC was beneficially only in the [<sup>18</sup>F]-MISO-avid, HPV-negative subset. These data demonstrate that hypoxic cells, although present, likely do not represent the treatment-limiting population in the HPV-positive subset of this disease.

Another distinction of the importance of hypoxia in disease subtypes was recently revealed in prostate cancer. An earlier study of 247 patients used oxygen needle electrodes to assess hypoxia and found that high levels are associated with rapid biochemical failure in patients treated with curative intent by radiotherapy (Milosevic *et al*, 2012). A follow-up study in this same patient cohort identified two distinct genetic subtypes and although hypoxia was present in both at similar levels, it was associated with poor prognosis only in the subtype with high genomic instability (Lalonde *et al*, 2014). This distinction was validated using a hypoxic gene signature in an independent cohort of patients treated by surgery alone. Again, one must conclude that like HNSCC, hypoxic cells in prostate cancer may be treatment-limiting in some patients and not in others.

This prostate study illustrates a second important context that should be considered when evaluating HAPs. In HNSCC, the association of hypoxia with local-regional failure is consistent with the direct resistance of hypoxic cells to radiation. However, in prostate and cervix cancer the influence of hypoxia on prognosis is associated with regional or distal metastasis (Milosevic *et al*, 2012). Hypoxia has been shown to drive properties associated with aggressive disease, including increased angiogenesis, genetic instability, metastasis, and stemness (Wouters and Koritzinsky, 2008). In these cases, there is a need to distinguish the role of hypoxia in contributing to disease progression from its role in limiting treatment response. Future personalised approaches to cancer therapy using HAPs will thus need to include not only assessment of hypoxia, but also an additional evaluation of the genetic subtypes and an understanding of the role of hypoxia in patient prognosis.

Finally, it is important to distinguish the use of HAPs in curative vs non-curative treatment settings. As discussed above, the benefit from HAPs in definitive regimens is expected to be limited to patients where hypoxic cells are in fact responsible for treatment failure. In non-curative settings, HAPs may prove to be beneficial in tumours even when this is not the case. Evofosfamide was developed as a chemotherapeutic agent that could exploit the presence of hypoxic cells in advanced cancers. The success of this approach is dependent on sufficient levels of hypoxia coupled with a bystander effect due to diffusion of the active effector to other cells in the tumour as discussed below. In this case the hypoxic cells may or may not be contributing to disease progression or prognosis. To date, evofosfamide has only been tested in this context and the potential benefit of this agent in curative combination settings remains unknown.

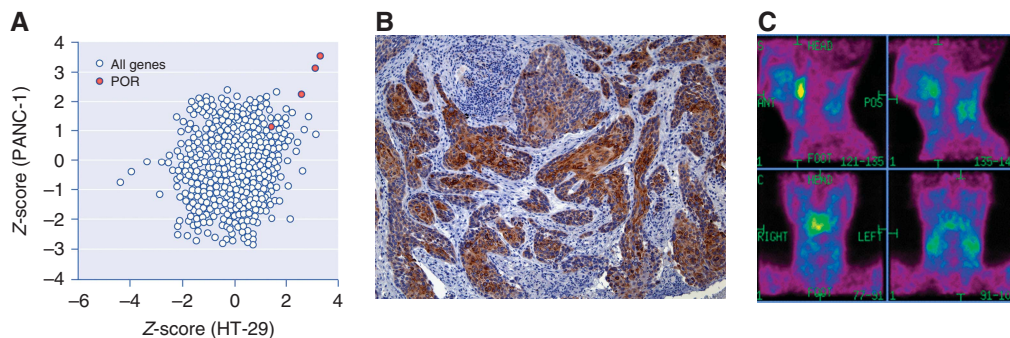
#### IDENTIFICATION AND PROFILING OF PRODRUG-ACTIVATING REDUCTASES

The requirement for enzymatic reduction of HAPs to facilitate their therapeutic effect (Figure 1) implies that the expression of activating reductases may be an important source of variability between individual tumours in sensitivity to HAPs. For this reason, significant effort has focused on identifying enzymes that are competent for HAP activation and on characterising their

expression in cancer. Most HAPs (including nitroaromatics, quinones and benzotriazine di-oxides) are activated via a mechanism that begins with one-electron reduction by flavin-dependent oxidoreductases to generate an oxygen-sensitive radical (Figure 1). Ninety annotated human flavoproteins catalyse two-electron transfer from NAD(P)H to flavin nucleotide cofactors, which can effect one-electron reduction of endogenous and xenobiotic substrates (Lienhart *et al*, 2013). HAPs with one-electron reduction potentials above  $\sim -400$  mV can compete with the flavin cofactors for electron acceptance and are activated in this manner (Wardman, 2001).

Identifying the specific flavoenzymes that are responsible for activation of HAPs has proven difficult in light of functional redundancy in the flavoproteome, in addition to the low-substrate specificities of these proteins as xenobiotic metabolising enzymes. Studies utilising recombinant proteins or forced expression in isogenic cell lines have identified a number of flavoproteins to be competent for HAP activation *in vitro*. These include POR, MTRR, NDOR1, NOS isoforms, FOXRED2 and CYB5R3 (Patterson *et al*, 1997; Papadopoulou *et al*, 2003; Guise *et al*, 2007; Chandor *et al*, 2008; Guise *et al*, 2012; Meng *et al*, 2012; Wang *et al*, 2014; Hunter *et al*, 2014b). However, these gain-of-function approaches do not necessarily implicate specific enzymes as major mediators of HAP activation at endogenous levels of expression. Emerging technology for efficient and cost-effective generation of definitive gene knockout models and loss-of-function genetic screens (Shalem *et al*, 2014) will facilitate a more precise description of this aspect of HAP pharmacology. Indeed, we recently utilised reductase-focused and genome-scale RNAi libraries to identify POR (P450 oxidoreductase) as the single most important modifier of sensitivity to the benzotriazine di-oxide HAP SN30000 in hypoxic cell culture (Figure 2A) (Hunter *et al*, 2015). Knockdown or knockout of *POR* significantly (but incompletely) abrogated SN30000 activity *in vitro*, and expression of *POR* correlated with activation of SN30000 and tirapazamine in a panel of diverse carcinoma cells. Importantly, we found *POR* to be highly expressed in a subset of HNSCC (Figure 2B) with much lower intratumour heterogeneity than the hypoxia marker carbonic anhydrase 9, suggesting that measuring *POR* expression by diagnostic biopsy may be a viable biomarker strategy. We also identified strong *POR* expression in some HNSCC found to harbour regions of hypoxia (Figure 2C); the coincidence of these two features implies a tumour phenotype likely to benefit from HAPs. A critical question is the extent to which reductase expression will influence tumour response to HAPs when weighed against other putative biomarkers such as hypoxia and modifiers of effector sensitivity. This question has been difficult to address, in part because the specific binding of 2-nitroimidazole bioreductive probes such as EF5, pimonidazole, FAZA, misonidazole or HX4 is also dependent on both hypoxia and one-electron reductase activity (Wang *et al*, 2012). Although confounding in the context of discriminating individual sensitivity determinants, the demonstration that EF5 and benzotriazine di-oxide HAPs are activated by the same reductases (Wang *et al*, 2012, 2014) suggests potential for 2-nitroimidazole PET imaging radiopharmaceuticals as dual reporters of both hypoxia and one-electron reductase activity, at least for this class of HAPs.

In contrast to the abovementioned agents, aliphatic (tertiary amine) N-oxides such as banoxantrone are activated via two-electron reduction that is catalysed by CYP isozymes. The basis for oxygen sensitivity in this mechanism is presumed to be competitive oxidation by O<sub>2</sub> of reducing centres in the CYP catalytic cycle. Some quinone and nitroaromatic HAPs are also substrates for obligate two-electron reductases that are not inhibited by oxygen. For example, the significant single-agent activity of PR-104 in H460 xenografts (Patterson *et al*, 2007) was subsequently found to be caused by strong expression of the two-electron reductase AKR1C3 in this model (Guise *et al*, 2010). Although such effects



**Figure 2.** HAP activation in tumours. **(A)** Identification of POR as a major determinant of SN30000 sensitivity by high-throughput shRNA screening of HT-29 and PANC-1 cells. The metric plotted is the Z-transformed  $\log_2$  of hairpin enrichment factor (treated/control) following exposure to SN30000. **(B)** Immunohistochemical staining for POR in a tissue section from an HPV-negative hypopharyngeal squamous carcinoma. Intense, relatively homogenous POR staining is observed in the malignant epithelium with minimal reactivity in the tumour-associated stroma. **(C)** Pre-treatment [ $^{18}\text{F}$ ]-misonidazole PET imaging for hypoxia in the same patient showing an avid mass in the posterior pharynx. Reproduced, with permission, from (Hunter *et al*, 2015).

can be considered an off-target liability, they may be exploitable in malignancies that strongly express the implicated two-electron reductase. The latter consideration has led to interest in evaluating PR-104 for the treatment of acute leukaemias (Houghton *et al*, 2011; Konopleva *et al*, 2015), some of which highly express AKR1C3 (Birtwistle *et al*, 2009; Jamieson *et al*, 2014). Indeed, Moradi Manesh *et al* (2015) recently identified strong AKR1C3 expression in primary paediatric T-lineage ALL xenografts, in which the AKR1C3-dependent activity of PR-104 was superior to the clinical combination of vincristine, dexamethasone and l-asparaginase. Although these findings point to an opportunity for PR-104, observations of hypoxia-related activity of HAPs in leukaemia models (Benito *et al*, 2015), presumably mediated by one-electron reduction, suggest a need for a more comprehensive set of predictive biomarkers in this setting.

#### DETERMINANTS OF INTRINSIC SENSITIVITY TO PRODRUG METABOLITES

The above determinants address the potential for HAPs to release antineoplastic agents (effectors) selectively within the tumour microenvironment. The next requirement is to identify biomarkers that predict sensitivity of the tumour cells to the effector, an issue that has received relatively little attention in the HAP context. For the DNA-reactive cytotoxics generated by most HAPs, DNA damage response (DDR) pathways will obviously be important although with differences between pharmacophores. Several studies have evaluated effects of knockout or knockdown of DNA repair genes on HAP sensitivity *in vitro*. Tirapazamine was shown to have a two- to five-fold higher potency against hypoxic human and Chinese hamster ovary (CHO) cells in which homologous recombination repair (HRR) genes (*BRCA1*, *BRCA2*, *Rad51d*, *Ercc1*, *Ercc4/Xpf*, *Xrcc2*, *Xrcc3*) were mutated or suppressed by RNAi (Evans *et al*, 2008). CHO cell lines with mutations in HRR genes (*Ercc1*, *Ercc4/Xpf*, *Rad51d*) also showed marked hypersensitivity to PR-104A and its active metabolite PR-104H (Gu *et al*, 2009), as also reported for evofosfamide, which showed increased potency against lines with defects in *Xrcc3*, *Ercc4/Xpf*, *BRCA1*, *Brca2* and *FANCA* (Meng *et al*, 2012).

Direct comparison of HRR dependence of different HAP classes showed cytotoxicity of prodrugs of DNA crosslinkers (PR-104A, evofosfamide) to be more dramatically potentiated by HRR defects than for benzotriazine di-oxides (tirapazamine, SN30000 and SN29751) (Hunter *et al*, 2012). This same pattern has been observed in human triple-negative breast and colon carcinoma

lines with suppressed HRR, including demonstration of notable activity of PR-104 and evofosfamide as monotherapy or in combination with radiotherapy against *BRCA2*-null DLD-1 xenografts (Hunter *et al*, 2014a). These findings point to the potential for use of HAPs such as evofosfamide against HRR-defective tumours, potentially including *BRCA*-related breast and ovarian carcinoma. A rational biomarker strategy may be use 2-nitroimidazole PET imaging to evaluate hypoxia plus one-electron reductase activity in conjunction with the assessment of HRR status by mutational profiling as validated clinically for PARP-1/2 inhibitors (Mateo *et al*, 2015). Although attractive in principle, further work is needed to validate DDR biomarkers specific to DNA crosslinking metabolites. It is disappointing, in that respect, that there has been little systematic effort to develop predictive biomarkers for crosslinking agents, other than mutation or expression of a single DNA repair gene (*ERCC1*) (Gao and Liu, 2015), despite the ongoing clinical importance of this class. Even for the most widely used crosslinker, cisplatin, biomarkers that are predictive of sensitivity or resistance are still not in routine clinical use (Bowden, 2014)—a problem that is symptomatic of a broader neglect of determinants of sensitivity to cytotoxic chemotherapy.

A central issue in developing biomarkers of intrinsic sensitivity is that of spatial heterogeneity of hypoxia within tumours, coupled with the allied question of whether the critical target population comprises only the hypoxic cells themselves. In relation to DDR biomarkers, it is noteworthy that persistent hypoxia transcriptionally or translationally downregulates most DNA repair pathways, including HRR (Scanlon and Glazer, 2015), which is thus expected to confer additional sensitivity to HAPs just as it does to conventional crosslinking agents (Chan *et al*, 2008) and PARP-1/2 inhibitors (Chan *et al*, 2010). However, although HAPs are designed for activation within hypoxic microenvironments, diffusion of effectors out of hypoxic zones has the potential to extend activity to cells that have not experienced hypoxic stress. This 'bystander effect', illustrated in Figure 3, has been suggested to contribute to the single-agent activity of PR-104 (Foehrenbacher *et al*, 2013) and evofosfamide (Sun *et al*, 2012) in tumour xenografts. Thus for HAPs with relatively stable effectors, intrinsic sensitivity biomarkers in theoxic compartment may be important determinants of response. Clear evidence for bystander effects of HAPs is provided by the killing of activation-deficient 'target' cells by activation-competent 'activator' cells in anoxic 3D multicellular layer (MCL) co-cultures; bystander killing is seen with dinitrobenzamide mustards related to PR-104 but not with tirapazamine (Wilson *et al*, 2007). In addition, the active metabolites PR-104H and PR-104M diffuse out of anoxic 3D multicellular layers exposed

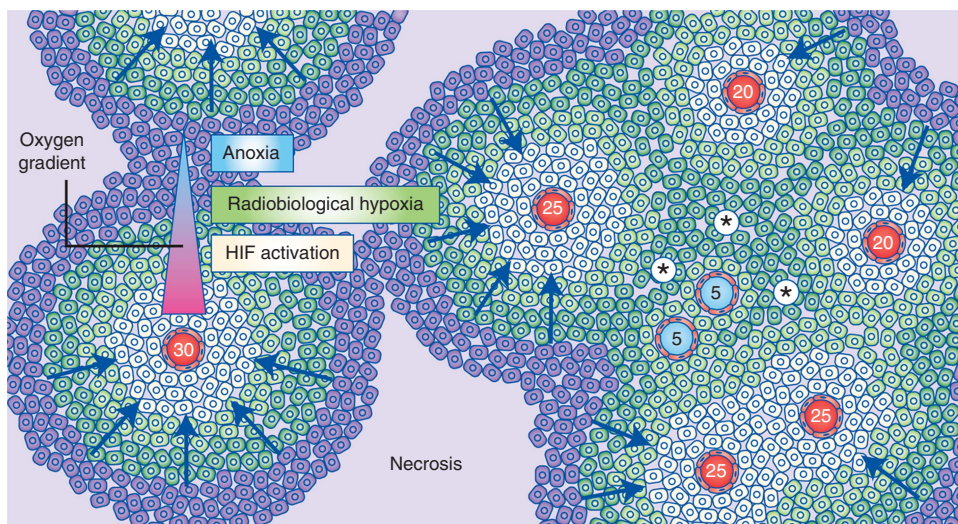


Figure 3. Hypothetical transverse section of tumour vascular chords showing steady state hypoxia gradients resulting from spatial heterogeneity of perfusion. Therapeutically significant zones are indicated in the oxygen concentration gradient, demarking cells that are classified as euoxic ( $O_2 > 25 \mu M$ ), moderately hypoxic ( $O_2 < 25 \mu M$ , typically sufficient to induce a HIF-1 response), radiobiologically hypoxic ( $O_2 < 5 \mu M$ ) and essentially anoxic ( $O_2 < 0.5 \mu M$ , enabling efficient activation of nitroaromatic HAPs) beyond which necrosis ensues. Numbers indicate intravascular  $O_2$  concentrations ( $\mu M$ ), which can vary temporally as well as spatially. Perivascular hypoxia is shown associated with two poorly perfused vessels in the centre of the section. Chemoresistant and radioresistant cells at moderate hypoxia ('HIF activation' + 'radiobiological hypoxia') are accessible to bystander effects resulting from diffusion of effectors from nitroaromatic HAPs (arrows), but only if contiguous with severely hypoxic (anoxic) regions. Hypoxic regions that are beyond the bystander range of nitroaromatic prodrugs (asterisks) may be more effectively treated by HAPs such as benzotriazine di-oxides that are less sensitively inhibited by oxygen.

to PR-104A at rates high enough to account for much of the monotherapy activity of PR-104 in xenograft models (Foehrenbacher *et al*, 2013).

This bystander effect is likely also to be important for killing of moderately hypoxic chemoresistant and radioresistant cells in tumours (Figure 3) given that nitroaromatic HAPs are activated only under severe hypoxia in cell culture (Koch, 1993; Hicks *et al*, 2007; Meng *et al*, 2012). However, exploiting hypoxia in this way depends on the chemo- and radioresistant hypoxic populations being contiguous with regions that are essentially anoxic. Given the macroregional variations in perfusion and hypoxia that are typical within tumours, many regions of moderate hypoxia (asterisks in Figure 3) will not meet this criterion. In such cases, direct targeting of moderately hypoxic cells with benzotriazine di-oxide HAPs (tirapazamine, SN30000) that are activated at ~10-fold higher  $pO_2$  than nitroaromatic HAPs (Hicks *et al*, 2007, 2010) may be a preferable strategy. These considerations point to a need to understand the spatial distribution of hypoxia and other key biomarkers in order to rationally select an optimal HAP for an individual tumour; this will likely call on emerging capabilities for functional imaging of intratumour heterogeneity (O'Connor *et al*, 2015a) and its integration with molecular profiling and histopathology.

## CONCLUSIONS

An emerging paradigm in cancer medicine is to personalise therapy using defined biomarkers, with small molecule and antibody-based therapeutics targeting known mutant oncogenes in individual tumours being the best examples. Identification and validation of predictive biomarkers for HAPs is a considerably more challenging prospect: determinants of sensitivity are multifactorial, and in most cases the primary target is a transient hypoxic subpopulation that limits treatment outcomes with current standards of care—although additional utility may be

achievable if bystander effects enable targeting of relatively well-oxygenated cells in tumours. Our current ability to identify specific clinical settings in which hypoxia can be leveraged for therapeutic advantage is still surprisingly weak, and the molecular profile of the key treatment-limiting subpopulations is incompletely understood. Despite these limitations, there have been important advances in relation to each aspect of the challenge, including development of high-spatial resolution hypoxia imaging in tumours by oxygen-enhanced MRI (O'Connor *et al*, 2015b) and the advent of efficient forward genetic screening for discovery of drug sensitivity genes using whole-genome CRISPR/Cas9 knockout libraries (Shalem *et al*, 2014). The latter can be expected to provide more complete, pharmacophore-specific, predictive biomarker sets to complement detection of tumour hypoxia.

One of the additional challenges in the clinical development of HAPs is that validation of predictive biomarkers depends on identifying responsive tumours, yet monotherapy activity based on conventional response endpoints is an unrealistic expectation in most cases. Thus, although not discussed in this short review, pharmacodynamic biomarkers that demonstrate activity in the critical target subpopulation (e.g. loss of hypoxic cells as assessed by functional imaging) are also needed to justify advancement of HAPs to combination settings. From that perspective, it is likely that the long-anticipated goal of overcoming (and exploiting) tumour hypoxia with prodrugs will require further development of both response biomarkers and predictive biomarkers, drawing on the increasingly powerful tools of molecular oncology. It is encouraging that first steps towards that goal are being taken in two multi-centre phase 2 trials of the hypoxia-activated pan-ERBB inhibitor tarloxotinib (NCT02454842 and NCT02449681) in which pre-treatment and post-treatment EGFR status and baseline HX4 PET imaging for hypoxia is being evaluated at select sites.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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