Chapter 6
Hypoxia-Directed Drug Strategies to Target the Tumor Microenvironment

Michael P. Hay, Kevin O. Hicks, and Jingli Wang

Abstract Hypoxia is an important component of the tumor microenvironment and has been the target of drug discovery efforts for almost half a century. These efforts have evolved from offsetting the impact of hypoxia on radiotherapy with oxygen-mimetic radiosensitizers to using hypoxia as a means to selectively target tumors. The more recent description of hypoxia-inducible factors and their role in the hypoxia response network has revealed a host of new drug targets to selectively target tumors. We are developing hypoxia-directed drugs in each of the following areas: novel radiosensitizers for hypofractionated radiotherapy, a second-generation benzotriazine di-N-oxide hypoxia-activated prodrug, and a hypoxia-inducible factor-1–dependent cytotoxin that targets glucose transport. These projects are discussed in the context of hypoxia-directed drug discovery.

Keywords Hypoxia • Drug discovery • Nitroimidazole • Radiosensitizer • Hypoxia-activated prodrug • Biomarker • Tirapazamine • SN30000 • HIF-1α • Glucose transport

6.1 Introduction

6.1.1 Hypoxia as a Therapeutic Target

Hypoxia initially arises as a consequence of oxygen consumption in small tumors or metastases. The cellular response to this hypoxia plays a significant role in the development of the tumor microenvironment and influences the expansion of tumor

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C. Koumenis et al. (eds.), *Tumor Microenvironment and Cellular Stress*, Advances in Experimental Medicine and Biology 772, DOI 10.1007/978-1-4614-5915-6_6,

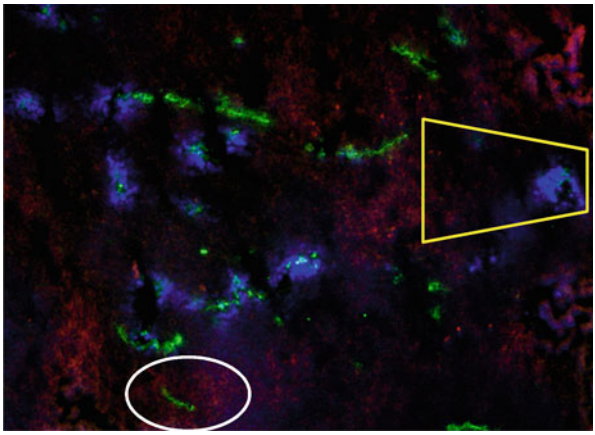

Fig. 6.1 Detection of hypoxic cells in a human colon cancer xenograft HCT116 grown subcutaneously in nude mice. *Yellow box* shows diffusion-limited hypoxia. *White oval* shows perfusion-limited hypoxia. Hypoxic marker EF5 (60 mg/kg) was administrated intraperitoneally 1.5 h and blood vessel perfusion marker Hochest33342 (40 mg/kg) was administered intravenously 2 min before the mice were killed. The tumor was removed immediately and frozen in octanol. Frozen sections (8 **μ**m) were immunostained for the hypoxic marker (EF5, *red*), blood vessels (CD1, *green*), and perfused blood vessels (Hochest33342, *blue*). (Jingli Wang, unpublished data)

vasculature, resulting in a disorganized, inefficient tumor microvascular network that has irregular blood flow (Jain 2005; Pries et al. 2009). In turn, this exacerbates existing hypoxia and leads to considerable heterogeneity in oxygen concentrations that may fluctuate spatially and temporally (Dewhirst et al. 2008) (Fig. 6.1). The characterization of hypoxia accordingly depends on the techniques used to measure it. Whereas fine-needle oxygen electrode measurements provide a direct gauge of oxygen tension and have demonstrated a wide range of oxygen concentrations in human tumors (Vaupel et al. 2007), the use of exogenous molecular probes such as 2-nitroimidazoles or endogenous markers such as downstream products of genes regulated by hypoxia-inducible factors (HIFs) report different levels of hypoxia (Fig. 6.2). Nitroimidazole probes are typically activated at levels of less than 1 μM of oxygen, whereas HIF-1 is stabilized at higher oxygen concentrations (Tuttle et al. 2007).

The majority of clinical studies have shown that hypoxia results in compromised outcomes across a wide range of diseases and treatment modalities (Horsman et al. 2012; Nordsmark et al. 2005; Vaupel and Mayer 2007). Both chronic hypoxia (Gray et al. 1953; Thomlinson and Gray 1955) and intermittent, or cycling, hypoxia within solid tumors can limit radiotherapy (Brown 1979). Poor perfusion and significant diffusion gradients exist within tumors (Dewhirst et al. 2008) that, along with high interstitial pressures (Heldin et al. 2004), can limit the diffusion of chemotherapeutic agents into hypoxic regions (Minchinton and Tannock 2006). This, when combined with a slowing of proliferation in these areas, can cause resistance to commonly used antiproliferative agents. Identification of the role of HIF-1 in the hypoxia response network (Semenza 2003) has revealed how hypoxia influences survival processes such as increased angiogenesis (Gnarra et al. 1996) and

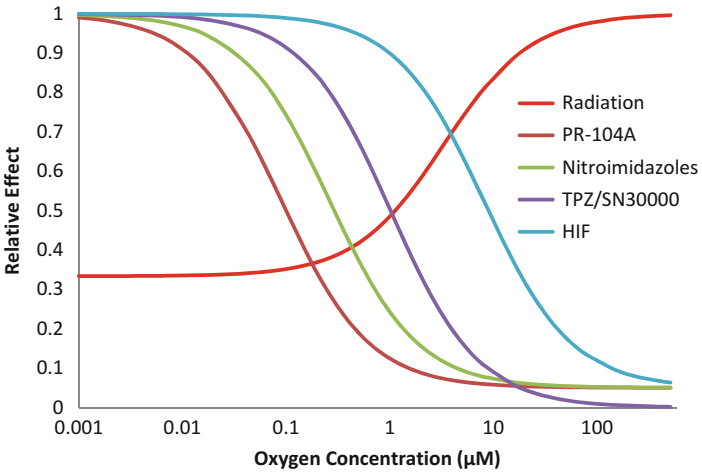

Fig. 6.2 Illustration of oxygen dependence for cellular response. Radiation sensitivity of clonogenic cell killing increases with increasing oxygen concentration, reaching half-maximal at approximately 4–5 μM (Wouters and Brown 1997), whereas it decreases for hypoxia-activated prodrugs PR-104 (Hicks et al. 2007) and tirapazamine/SN30000 (Hicks et al. 2004, 2010). For nitroimidazoles, oxygen dependence of intracellular binding of EF5 (Tuttle et al. 2007) is similar to the oxygen dependence of the sensitizer enhancement ratio of misonidazole (Carlson et al. 2011). Stabilization of the hypoxia-inducible factor generally occurs under more moderate hypoxic conditions (Tuttle et al. 2007)

vasculogenesis (Kioi et al. 2010), resistance to cell death (Graeber et al. 1996), aerobic glycolysis (Semenza 2010), and genomic instability (Bindra et al. 2007; Huang et al. 2007). Furthermore, the contribution of tumor hypoxia to invasiveness and metastasis (Chang et al. 2011; Hill et al. 2009) potentially compromises a third treatment modality: surgery. The prevalence of tumor hypoxia, combined with its effect on tumor survival, progression, and resistance to therapy, marks hypoxia as a compelling target for current drug discovery efforts.

6.1.2 Drug Development

In tandem with the growing understanding of the effect of hypoxia, an evolving series of drug discovery efforts have sought to overcome or leverage the effects of hypoxia for therapeutic gain (Denny 2010; Semenza 2007; Wilson and Hay 2011). The major drug discovery effort was centered on chemical radiosensitizers and spanned several decades (Wardman 2007), but minimal clinical success resulted in dwindling efforts in this field (Overgaard 2007).

A paradigm shift in hypoxia targeting occurred in the mid-1980s. Rather than minimizing the impact of hypoxia on radiotherapy, a new strategy sought to use hypoxia as a physiological target that could promote activation of prodrugs to kill tumor cells. Hypoxia-activated prodrugs (HAPs) have been extensively explored

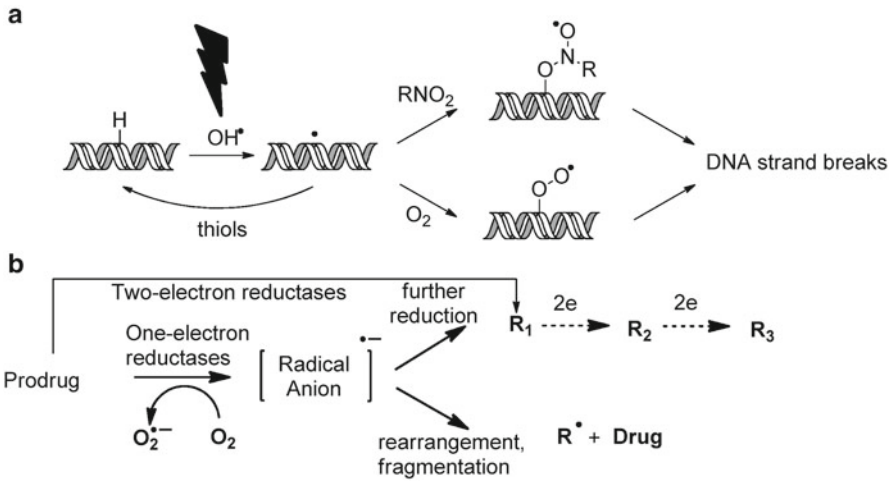

Fig. 6.3 (**a**) Mechanism of oxygen-mimetic radiosensitization by electron-affinic nitroaryl radiosensitizers. (**b**) Mechanism of hypoxia-activated prodrugs

over several decades (Brown and Wilson 2004; Chen and Hu 2009; McKeown et al. 2007; Rockwell et al. 2009; Wilson and Hay 2011).

Identification of the HIF as the "master regulator" of hypoxic response and a key drug target (Giaccia et al. 2003; Semenza 2003) resulted in the discovery of a plethora of small molecules that could be potential HIF inhibitors (Xia et al. 2012). However, much of this work has had limited application. Many agents identified as HIF-1α inhibitors actually target upstream or downstream components in the HIF response network or have pleiotropic effects. In addition, these agents are not necessarily cytostatic or cytotoxic, nor are they necessarily selective for hypoxic cells.

Overall, few agents developed to target hypoxia have been registered. Tumor hypoxia has been a niche area, predominantly the preserve of academic groups, and only with the elaboration of the hypoxia response network has hypoxia received mainstream attention as a validated target for drug development.

6.1.3 Defining the Hypoxic "Target"

Definition of the "target" depends on the approach directed against the hypoxic cells. For oxygen-mimetic radiosensitizers the target is a DNA radical generated by ionizing radiation (Fig. 6.3a), and selectivity results from competition between oxygen and the electron-affinic nitroimidazole group for this radical. Since oxygen is vastly more efficient at scavenging these radicals, nitroimidazoles effectively sensitize only hypoxic cells.

A more complex situation exists for HAPs (Fig. 6.3b). The prodrug is reduced by a one-electron reductase to form a radical anion. This radical anion may be "scavenged" by oxygen to reproduce the prodrug with production of superoxide. In the

absence of oxygen the radical anion may undergo a variety of transformations, depending on chemical class, leading to the activated drug. Reduction of the prodrug by two-electron reductases removes the potential for back-oxidation of the radical anion, leading to a loss of hypoxic selectivity. As a consequence, the target is the intersection of three elements: hypoxia, enzymes to activate the prodrug, and intrinsic sensitivity to the activated drug. The initial concept invoked tumor hypoxia as unique to tumor tissue and, essentially, a binary switch, suggesting it is an ideal drug target for HAPs (Denny et al. 1996). However, hypoxia exists in normal tissues, and tumor cells at intermediate oxygen tension are important for tumor progression (Wouters and Brown 1997), making hypoxia a more complex target. The second component requires the location of appropriate enzymes to activate the prodrug within the tumor. NADPH:cytochrome P450 oxidoreductase has been identified as a key one-electron reductase responsible for the activation of many HAPs (Guise et al. 2007; Meng et al. 2012; Patterson et al. 1998; Wang et al. 2012b), but contributions from other one-electron reductases (e.g., aldehyde oxidase, xanthine oxidase, nitric oxide synthases, thioredoxin reductase, NADH-dependent cytochrome b5 reductase, methionine synthase reductase, and NADPH-dependent diflavin oxidoreductase) also have been reported (Adams and Rickert 1995; Ask et al. 2003; Cenas et al. 2006; Chandor et al. 2008; Guise et al. 2012; Papadopoulou et al. 2003; Patterson et al. 1998; Tatsumi et al. 1986; Ueda et al. 2003). The expression of these enzymes and their relative contributions to HAP activity across human tumors is incompletely understood; however, significant variations between cell lines (Guise et al. 2012; Wang et al. 2012b) and individual human tumors (Evans et al. 2000; Patterson et al. 1997) has been demonstrated. The electron affinity of the prodrug seems to be the key determinant of activation (Wardman 2001), indicating little substrate specificity for most of the one-electron reductases. The third constituent of the HAP target is the intrinsic sensitivity of the target cells to the activated drug. Strategies using prodrugs that release cytotoxins that cross-link DNA will be dependent on DNA repair status and may cause normal tissue toxicity if activated inappropriately. Prodrugs delivering inhibitors of specific molecular targets, such as the human epidermal growth factor receptor, have been reported more recently (Patterson et al. 2009). For these agents, the relative expression of the molecular target in hypoxic and normal tissues contributes to the overall target. In the case of HIF inhibitors, the molecular targets are structurally diverse, and hypoxic selectivity is provided by the level of overexpression under hypoxic conditions relative to the levels of the target in normal tissue and the specificity of the inhibitor for the particular molecular target.

6.2 Radiosensitizers

6.2.1 Introduction

Attempts to offset the negative effects of hypoxia on radiation therapy initially focused on manipulation of tumor oxygen status (e.g., fractionated radiation schedules to allow reoxygenation between fractions [Kallman and Dorie 1986]; hyperbaric

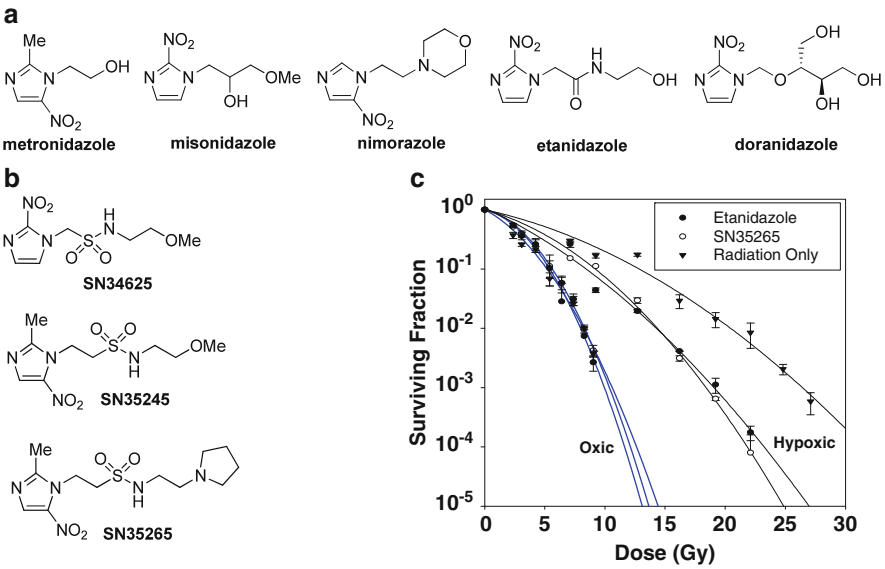

Fig. 6.4 (**a**) Clinically investigated nitroimidazoles. (**b**) Representative novel 2- and 5-nitromidazole sulfonamides. (**c**) Clonogenic survival curves of HCT116 colorectal carcinoma cells after increasing dose of radiation in oxia (*blue*) and anoxia (*black*) in the presence of equitoxic doses of etanidazole (1 mM) and SN35265 (0.7 mmol)

oxygen treatment [Bennett et al. 2012; Overgaard and Horsman 1996], and nicotinamide in combination carbogen breathing with accelerated radiotherapy [ARCON] [Janssens et al. 2012; Kaanders et al. 2002]). Drug discovery efforts have been centered on the development of chemical radiosensitizers and, in particular, oxygen-mimetic sensitizers.

6.2.2 Nitroimidazole Oxygen Mimetics

The concept of an electron-affinic nitroaryl molecule as a radiosensitizer in hypoxic tumor tissue has a long history (Dische 1985, 1991; Wardman 2007). The 5-nitroimidazole antibiotic metronidazole (Fig. 6.4) was identified as an effective radiosensitizer (Asquith et al. 1974) and displayed clinical benefit (Urtasun et al. 1976). More electron-affinic 2-nitroimidazoles were explored, and misonidazole was identified and advanced to clinical trials (Adams et al. 1976). Misonidazole underwent extensively trials with fractionated radiotherapy; despite indications of clinical benefit (Overgaard 1994), delayed peripheral neuropathy limited treatment (Grigsby et al. 1999; Saunders and Dische 1996). The electron affinity of the nitroimidazole group is the key parameter for radiosensitization and toxicity (Adams et al. 1979a, b). Thus, 5-nitroimidazoles with lower electron affinity had lower

toxicity and larger doses could be used to offset their weaker radiosensitization. This led to the identification of nimorazole as a radiosensitizer (Overgaard et al. 1982, 1983) that is well tolerated (Overgaard et al. 1998; Timothy et al. 1984) and used clinically, but only in Denmark. Nimorazole is currently undergoing a phase III clinical trial with accelerated radiotherapy (Overgaard 2012).

Attempts to design more polar analogs with reduced lipophilicity and increased systemic clearance to minimize the neurotoxicity observed with misonidazole led to the development of etanidazole (Brown et al. 1981) and doranidazole (Murata et al. 2008; Oya et al. 1995). This approach was only partially successful: Etanidazole had reduced neurotoxicity compared to misonidazole (Coleman et al. 1990) but failed to provide benefit in head and neck cancer (Eschwege et al. 1997; Lee et al. 1995). Doranidazole is currently under investigation for pancreatic cancer (Karasawa et al. 2008) and non-small-cell lung carcinoma (Nishimura et al. 2007).

It is salutary to note that although nitroimidazole radiosensitizers have been extensively investigated clinically and that hypoxic modification was shown to be effective in a meta-analysis (Overgaard 2011), only nimorazole is in clinical use. Two main factors have contributed to the limited clinical success of radiosensitizers. Their use with fractionated radiotherapy – where fractionation of the radiation dose is designed to allow tumor reoxygenation between radiation fractions – reduces the potential for radiosensitization (Hill 1986; Kallman 1972). Fractionated radiotherapy ideally requires a dose of radiosensitizer with each fraction of radiation, a schedule that was unachievable with early 2-nitroimidazoles because of cumulative peripheral neurotoxicity. Perhaps most significant is that many of the trials were small and were conducted without prospectively identifying patients with hypoxic tumors, despite considerable heterogeneity in the level and extent of tumor hypoxia among patients (Hoogsteen et al. 2009).

However, the development of stereotactic body radiotherapy (SBRT) may offer a new opportunity for this class. SBRT uses hypofractionated (one to five doses), high-dose (25–60 Gy in total dose) radiation to treat primary tumors and oligometastases. Initial clinical results of using SBRT to treat a variety of primary tumors suggest locoregional control and toxicity profiles that compare to or improve on those of fractionated radiotherapy (Lo et al. 2010). Prospective, randomized trials to confirm these results compared to standard care will drive increasing use of SBRT. In addition, reduced treatment time and fewer patient visits, combined with emerging potential to replace surgery in patients for whom an outpatient procedure presents risk, indicates potential economic health advantages for SBRT. However, SBRT may accentuate the role of hypoxia in radioresistance because of the reduced opportunity for tumor reoxygenation during therapy (Brown et al. 2010; Carlson et al. 2011). This would offer the possibility of a renaissance for nitroimidazole radiosensitizers in conjunction with SBRT. A recent small, phase III trial of doranidazole in conjunction with intraoperative radiotherapy (25 Gy) for pancreatic tumors demonstrated a survival advantage (Nishimura et al. 2007).

Nevertheless, several barriers exist in the development of radiosensitizers for use with SBRT. Limited (doranidazole) or expired (misonidazole, etanidazole, nimorazole) patent protection for clinically evaluated nitroimidazoles will limit their

application, while the wide range of analogs prepared across the field restricts discovery of novel, patentable nitroimidazoles. The other challenge for future development of such radiosensitizers is the use of a biomarker to prospectively identify hypoxia in patients (See Sect. 6.5).

In addressing these challenges, we have recently identified a new class of nitroimidazole with a sulfonamide side chain, providing chemical novelty (Bonnet et al. 2012). A series of 2- and 5-nitroimidazole analogs have been designed and synthesized, and preliminary results show that representative compounds (Fig. 6.4b) produce comparable in vitro radiosensitization to etanidazole at nontoxic concentrations in hypoxic HCT-116 human colorectal carcinoma cells (Fig. 6.4c). The electron affinity of these compounds, as measured by one-electron reduction potential, is higher than corresponding 2- and 5-nitroimidazoles because of the influence of the strong electron-withdrawing side chain and results in increased radiosensitization. Metabolism is also increased in the more electron-affinic examples, resulting in hypoxia-selective cytotoxicity. This novel series provides the opportunity to leverage 30 years of drug development around the class and develop a third-generation radiosensitizer while including extravascular transport (EVT) (See Sect. 6.3.5) and hypoxia biomarker studies (See Sect. 6.5) early in the drug design process.

6.2.3 Molecular Targets in DNA Repair as Radiosensitizers

The targeting of DNA repair for radiosensitization using antimetabolites (Brown et al. 1971) is well established, although these agents work through multiple mechanisms (Shewach and Lawrence 2007). A range of histone deacetylation inhibitors also radiosensitize tumor cells through modulation of the DNA damage response (Camphausen and Tofilon 2007). Specific DNA repair proteins such as poly(ADP-ribose) polymerase (PARP) (Chalmers et al. 2010), ataxia telangiectasia mutated (ATM) pharmacokinetics (Sarkaria and Eshleman 2001), ATM- and Rad3-related (ATR) pharmacokinetics (Wang et al. 2004), and DNA-dependent pharmacokinetics (Blunt et al. 1995) are potential targets for radiosensitization (Helleday et al. 2008; Begg et al. 2011). PARP inhibitors can radiosensitize tumors (Albert et al. 2007; Calabrese et al. 2004), although some of their activity may be due to a vascular effect that results in reduced intermittent hypoxia (Senra et al. 2011). A range of PARP inhibitors are in clinical development and offer potential as radiosensitizers. Novel ATM inhibitors (KU55933 [Hickson et al. 2004] and KU60019 [Golding et al. 2009]) and ATR inhibitors (NU6027 [Peasland et al. 2011] and VE821 [Charrier et al. 2011; Reaper et al. 2011]) display radiosensitization in vitro (Pires et al. 2012). The selective DNA pharmacokinetics inhibitor NU7441 can radiosensitize tumor cells in vitro and in vivo (Zhao et al. 2006), whereas IC87361 (Kashishian et al. 2003) was reported to enhance radiation-induced delay in the growth of Lewis lung carcinomas (Shinohara et al. 2005). One concern about this approach is the potential for these agents to radiosensitize normal tissue within the radiation field. Although particular diseases may be

identified to provide synthetically lethal combinations (e.g., BRCA1 loss of function in combination with PARP inhibitors), another approach is to selectively target these agents to hypoxic tissues using a prodrug approach (Parveen et al. 1999; Cazares-Korner et al 2013).

6.3 Hypoxia-Activated Prodrugs

6.3.1 Introduction

HAPs (also called bioreductive prodrugs or hypoxia-selective cytotoxins) can be grouped into six classes based on their activation chemistry (Fig. 6.5). Quinone prodrugs such as EO9, based on the reductive activation of mitomycin C, were the first class to be explored (Lin et al. 1972; Phillips et al. 2013). The observation that redox cycling could provide a basis for hypoxia-selective cytotoxicity of nitroaryl compounds (Mason and Holtzman 1975) was followed by observations that some nitroimidazole radiosensitizers were also selectively toxic to hypoxic tumor cells in culture (Hall and Roizin-Towle 1975; Mohindra and Rauth 1976). This led to extensive studies of nitroheterocycles as hypoxia-activated prodrugs (Jenkins et al. 1990; Naylor et al. 1990; Threadgill et al. 1991), culminating in the bifunctional prodrug RB-6145 (Naylor et al. 1993), in which an alkylating bromoethylamine side chain increased cytotoxic potency (Hill et al. 1986). Clinical development of RB-6145 and its R-enantiomer (CI-1010) (Cole et al. 1992) was halted because of retinal toxicity

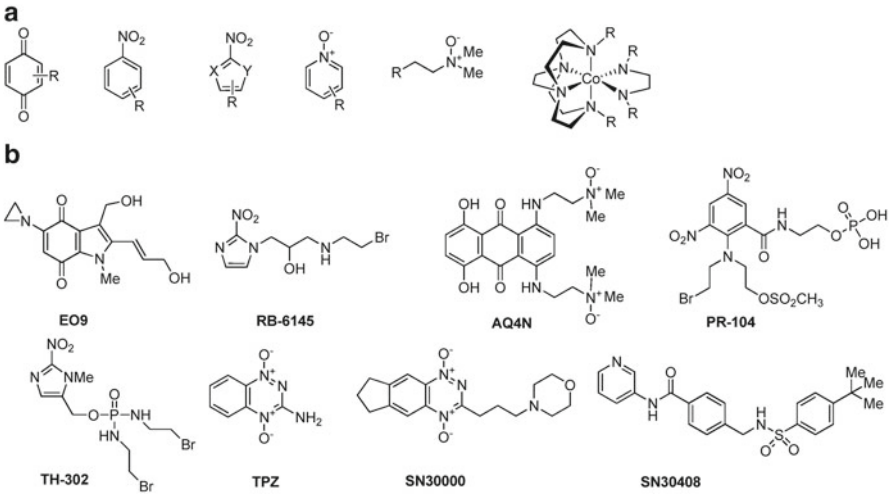

Fig. 6.5 (**a**) Main chemical classes used for hypoxia-activated prodrugs (HAPs). (**b**) Examples of HAPs

in preclinical models, providing early evidence that hypoxia in normal tissues could result in dose-limiting toxicities (Breider et al. 1998; Lee and Wilson 2000).

Description of the principles of bioreductive activation of nitroaryl prodrugs of nitrogen mustard (Denny and Wilson 1986) laid the groundwork for the eventual discovery of PR-104 as a HAP (Patterson et al. 2007). The hypoxic selectivity of aromatic N-oxides based on the 1,2,4-benzotriazine system led to the identification of tirapazamine (TPZ) (Brown 1993). Aliphatic N-oxides were shown to compete with oxygen for reduction by two-electron reductases, providing a mechanism for masking the DNA binding of DNA intercalators (Patterson 1993; Wilson et al. 1992), such as AQ4N (banoxantrone). Stable transition metal complexes (e.g., Co[III] [Milbank et al. 2009; Ware et al. 1993; Yamamoto et al. 2012] and Cu[II] [Parker et al. 2004]) can undergo hypoxia-selective, one-electron reduction to relatively unstable complexes (e.g., Co[II] and Cu[I]), releasing a cytotoxic agent. A vast assortment of compounds from these classes has been explored in the laboratory but only a handful have been evaluated clinically. Several of these provide informative examples of the challenges facing HAP discovery and are briefly discussed below.

6.3.2 PR-104

PR-104 arose from the structural optimization of simple nitroaryl nitrogen mustards (Denny and Wilson 1986) to selectively activated, diffusible mustard cytotoxins (Denny and Wilson 1993) and involved several design challenges. Elevation of the electron affinity of the 5-nitro group into a range suitable for bioreduction required additional electron-withdrawing substituents (e.g., a 3-NO_2 group) (Palmer et al. 1992). The relative arrangement of the four substituents provides the best combination of potency and hypoxic selectivity (Palmer et al. 1996). Addition of a carboxamide-linked solubilizing side chain (Palmer et al. 1994), combined with a phosphate prodrug approach, provides sufficient aqueous solubility.

The phosphate group is readily cleaved in plasma (Patel et al. 2007), and the nitro group then undergoes one-electron reduction to a nitro radical anion (Guise et al. 2007) (Fig. 6.6a), which is converted back to the prodrug in the presence of oxygen by redox cycling. Further reduction of the radical anion produces a nitrosobenzene that may undergo subsequent reduction to electron-donating hydroxylamine (PR-104H) and aminobenzene (PR-104M). These activated species cross-link DNA, forming cytotoxic lesions (Gu et al. 2009; Patterson et al. 2007; Singleton et al. 2009). PR-104 is activated under low oxygen concentrations (Hicks et al. 2007) (Fig. 6.2), but reduced species are sufficiently lipophilic and stable to diffuse from the cell of activation to surrounding tumor cells, known as the "bystander effect" (Foehrenbacher et al. 2013; Patterson et al. 2007; Wilson et al. 2007).

PR-104 displayed excellent in vitro hypoxic selectivity (6- to 160-fold), with single-agent activity and potentiation of radiation in SiHa, HT29, and H460 tumor xenografts (Patterson et al. 2007). PR-104 advanced to clinical trials (Jameson et al. 2010; McKeage et al. 2011), but normal tissue toxicity in humans prevented trials

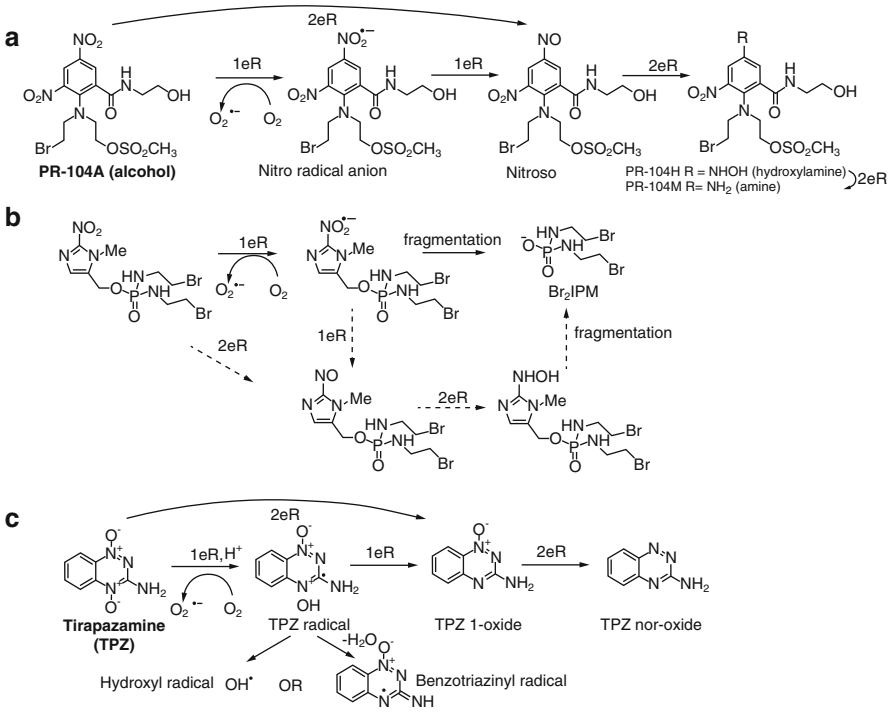

Fig. 6.6 Mechanism of activation of leading hypoxia-activated prodrugs. (**a**) PR-104. One-electron reduction of PR-104A to the nitro radical anion is reversed in the presence of oxygen. Under hypoxia, further reduction of the radical anion leads sequentially to the deactivated nitroso and the activated hydroxylamine (PR-104H) and amine PR-104M. Two-electron reduction of PR-104A bypasses the nitro radical anion and is not hypoxia selective. (**b**) TH-302. One-electron reduction under hypoxia leads to a radical anion. Radiolytic studies have demonstrated direct fragmentation of the radical anion to release the bromo-phosphoramidate mustard (Br_2-IPM). An alternate, stepwise two-electron reduction to the 2-hydroxylamine and subsequent fragmentation has been previously proposed. (**c**) Tirapazamine. One-electron reduction gives an *N*-oxide radical that may be reoxidized by oxygen. Under hypoxia, protonation and then rearrangement produces a carbon-centered tirapazamine (TPZ) radical. This TPZ radical may then eliminate water to give a DNA-damaging benzotriazinyl nitrogen-centered radical or release a hydroxyl radical. Further reduction of the TPZ radical, or two-electron reduction of TPZ, leads to the relatively nontoxic 1-oxide and nor-oxide. An analogous activation mechanism has been proposed for the related benzotriazine dioxide SN30000

from reaching an efficacious dose (Patel et al. 2011). Activation of PR-104 by the oxygen-insensitive two-electron reductase aldo-ketoreductase AKR-1C3 (Guise et al. 2010), was subsequently suggested as a factor contributing to this toxicity. A new strategy to leverage the presence of both hypoxia and AKR-1C3 expression in particular tumor types, including advanced leukemia (Houghton et al. 2011; Benito et al. 2011), has led to subsequent clinical trials (www.ClinicalTrials.gov identifier NCT01037556).

6.3.3 TH-302

A versatile prodrug strategy based around the 2-nitroimidazole-5-methanol moiety was able to release enediynes (Hay et al. 1999), aspirin (Everett et al. 1999), and a PARP inhibitor (Parveen et al. 1999) in a hypoxia-selective manner; nitroheterocyclic prodrugs of phosphoramidate mustards were shown to release cytotoxins upon reduction (Borch et al. 2000, 2001). These studies were a precursor to the discovery of TH-302, a 2-nitroimidazole-5-methyl phosphoramidite, as a HAP with excellent hypoxic selectivity (Duan et al. 2008; Meng et al. 2012). Steady-state and pulse radiolysis methods showed that TH-302 undergoes one-electron reduction and fragmentation to release bromo-isophosphoramide mustard (Meng et al. 2012) but did not exclude the initially proposed stepwise reduction of 2-nitroimidazole prodrugs to hydroxylamine or amine and fragmentation via an iminomethide (Borch et al. 2001) (Fig. 6.6b). The increased toxicity observed in cells that overexpress bacterial nitroreductase provides evidence of the potential for oxygen-insensitive, two-electron reduction and release of bromo-isophosphoramide mustard (Meng et al. 2012). The released mustard generates DNA cross-links that are responsible for hypoxic cytotoxicity (Meng et al. 2012). Extensive preclinical studies have shown the antitumor activity of TH-302 – either as a single agent (Sun et al. 2012) or in combination with commonly used chemotherapeutic drugs (Liu et al. 2012) and radiation (Lohse et al. 2012) – in many animal xenograft models. The anticancer efficacy of TH-302 correlated well with the levels of xenograft tumor hypoxia, confirming the hypoxic specificity of drug action in vivo (Lohse et al. 2012; Sun et al. 2012). TH-302 is currently the most advanced HAP in clinical development. Promising outcomes from phase II clinical trials (Borad et al. 2012; Chawla et al. 2011) led to the commencement of two randomized, placebo-controlled, phase III trials: one with TH-302 in combination with doxorubicin for advanced soft tissue sarcoma and the other in combination with gemcitabine for advanced pancreatic cancer.

6.3.4 Tirapazamine

TPZ (tirazone) is the prototypic example of a heterocyclic N-oxide HAP and dominated the field for almost two decades (Brown 1993, 2010; Denny and Wilson 2000). TPZ shows highly selective killing in cell culture under hypoxic compared to aerobic conditions (Zeman et al. 1986) as a result of rapid bioreductive metabolism (Baker et al. 1988; Hicks et al. 2003; Siim et al. 1996). One-electron reduction by, for example, NADPH:cytochrome P450 oxidoreductase (Fitzsimmons et al. 1994; Patterson et al. 1997, 1998), inducible nitric oxide synthase (Chinje et al. 2003), or nuclear localized reductases (Evans et al. 1998) produces a N-centered radical (Baker et al. 1988; Laderoute et al. 1988) that is efficiently back-oxidized to TPZ by oxygen (Fig. 6.6c). In the absence of oxygen, protonation and rearrangement leads

to an oxidizing radical (Anderson et al. 2003; Shinde et al. 2009, 2010; Yin et al. 2012) or hydroxyl radical (Chowdhury et al. 2007; Daniels and Gates 1996), both of which have been proposed as the species that damages cytotoxic DNA. DNA damage measured by comet assay (Olive et al. 1996; Siim et al. 1996) or induction of γH2AX (Olive et al. 2004; Wang et al. 2012b) correlates with the rates of bioreduction and reductase expression (Wang et al. 2012b) and is repaired by multiple mechanisms, including homologous recombination repair of double-strand breaks (Evans et al. 2008; Hunter et al. 2012). The radical species are short-lived and do not contribute to the killing of surrounding cells. Despite the lack of the bystander effect, TPZ is able to kill cells at intermediate oxygen concentrations because of activation at relatively high oxygen concentrations, with K-values (oxygen concentration for half-maximal hypoxic potency) in the range 1–3 μM (Hicks et al. 2004, 2007; Koch 1993), resulting in good complementarity with radiation (Hicks et al. 2004, 2007; Koch 1993; Wouters and Brown 1997), (Fig. 6.2). In contrast to PR-104 and TH-302, the two- and four-electron reduction products are markedly less cytotoxic than the parent drug (Baker et al. 1988), but this unproductive metabolism reduces potency.

Xenograft studies demonstrated cell killing complementing that of single-dose (Zeman et al. 1988) and fractionated radiation (Brown and Lemmon 1990, 1991). TPZ also demonstrated synergy with cisplatin in preclinical tumor models (Dorie and Brown 1993, 1994), resulting from hypoxia-dependent inhibition of cisplatin DNA cross-link repair (Kovacs et al. 1999).

TPZ has been intensively studied in clinical trials in combination with radiation and chemotherapy in head and neck (Rischin et al. 2005, 2010b), non-small-cell lung (Sandler et al. 2000; Shepherd et al. 2000; von Pawel et al. 2000; Williamson et al. 2005) and cervical carcinomas (Aghajanian et al. 1997; Covens et al. 2006; Craighead et al. 2000; DiSilvestro et al. 2012; Maluf et al. 2006; Rischin et al. 2010a) and has been extensively reviewed (Ghatage and Sabagh 2012; McKeown et al. 2007; Reddy and Williamson 2009). TPZ was well tolerated in early phase trials at doses resulting in plasma drug concentrations in the therapeutic range (Johnson et al. 1997; Senan et al. 1997). Early trials produced signs of activity with the initial phase III trial of TPZ/cisplatin in advanced non-small-cell lung cancer, demonstrating increased overall survival relative to cisplatin and radiation alone (von Pawel et al. 2000). This indication of activity was not confirmed in larger, randomized phase III trials in head and neck (Rischin et al. 2010b) and cervical carcinomas (DiSilvestro et al. 2012), and further development of TPZ has been halted.

Several issues were identified as affecting the efficacy of TPZ as a HAP. TPZ demonstrated significant toxicities that limited the therapeutic ratio (Ghatage and Sabagh 2012; McKeown et al. 2007; Reddy and Williamson 2009). TPZ also has low solubility, which required long infusion times (Graham et al. 1997; Senan et al. 1997). In addition, preclinical studies demonstrated that TPZ is substantially less selective for hypoxic cells in three-dimensional (3D) culture (Durand and Olive 1992) or xenografts (Durand and Olive 1997) than in monolayer cell culture, a consequence of limited EVT (Hicks et al. 1998).

6.3.5 *Discovery of a Second-Generation Benzotriazine Dioxide (SN30000)*

With these issues in mind we embarked on the discovery of a second-generation benzotriazine dioxide (BTO) as a HAP. Our aim was to identify TPZ analogs with superior activity against hypoxic cells in tumors by improving the solubility-potency product, hypoxia selectivity, and EVT using the end point of improved therapeutic activity in preclinical xenograft models at equivalent toxicity. It also was necessary to identify chemically novel compounds to secure an intellectual property position to support development.

A limited number of TPZ analogs had been prepared and evaluated (Kelson et al. 1998; Minchinton et al. 1992; Zeman et al. 1989), and little information on structure-activity relationships (SARs) existed. We prepared an initial toolset of 42 compounds with a range of substituents to explore SARs and we confirmed the positive relationship between the one-electron reduction potential, E(1), and anoxic potency in both clonogenic and growth inhibition (IC_{50}) assays (Hay et al. 2003).

EVT was investigated using multicellular layers (MCLs), a model of the tumor extravascular compartment in which cells are grown on porous support membranes in culture inserts submerged in culture medium (Cowan et al. 1996; Minchinton et al. 1997) and form diffusion-limited structures with central hypoxia (Hicks et al. 1998). Anoxia reduced TPZ transport in MCLs (Hicks et al. 1998, 2003; Kyle and Minchinton 1999), and reaction diffusion modeling using measured TPZ diffusion coefficients and rate constants for anoxic metabolism predicted steep gradients of TPZ in hypoxic tumor tissue, resulting in reduced cell killing. A spatially resolved pharmacokinetic/pharmacodynamic model for HT29 MCLs incorporating cytotoxic potency measured in anoxic cell cultures predicted increased resistance to TPZ in anoxic MCLs compared to stirred suspensions (Hicks et al. 2003). This confirmed that multicellular resistance to TPZ in anoxic 3D culture was primarily a result of limited transport and was responsible for the reduced efficacy of TPZ in 3D models (Durand and Olive 1992, 1997). This model was extended to tumors by incorporating the measured oxygen dependence (K-curve) of TPZ metabolism (Hicks et al. 2004) and measured TPZ plasma pharmacokinetics to simulate TPZ transport in a mapped microvascular network (Hicks et al. 2006). The model predicted that cell killing by TPZ in the hypoxic region is reduced relative to that achievable with no EVT limitation. In addition, the model successfully predicted activity of TPZ and 15 analogs from the SAR toolset in HT29 xenografts using measured plasma pharmacokinetics, transport parameters, and anoxic cytotoxicity (Hicks et al. 2006).

We also used the molecular toolset to investigate the SAR for transport, demonstrating that diffusion coefficients in HT29 MCLs increased with increasing $logP_{7.4}$ and decreased with molecular weight, number of hydrogen bond donors, and acceptors (Pruijn et al. 2005, 2008).

After developing the tools to efficiently evaluate novel BTO analogs, we used the screening method guided by the pharmacokinetic/pharmacodynamic model (Fig. 6.7a)

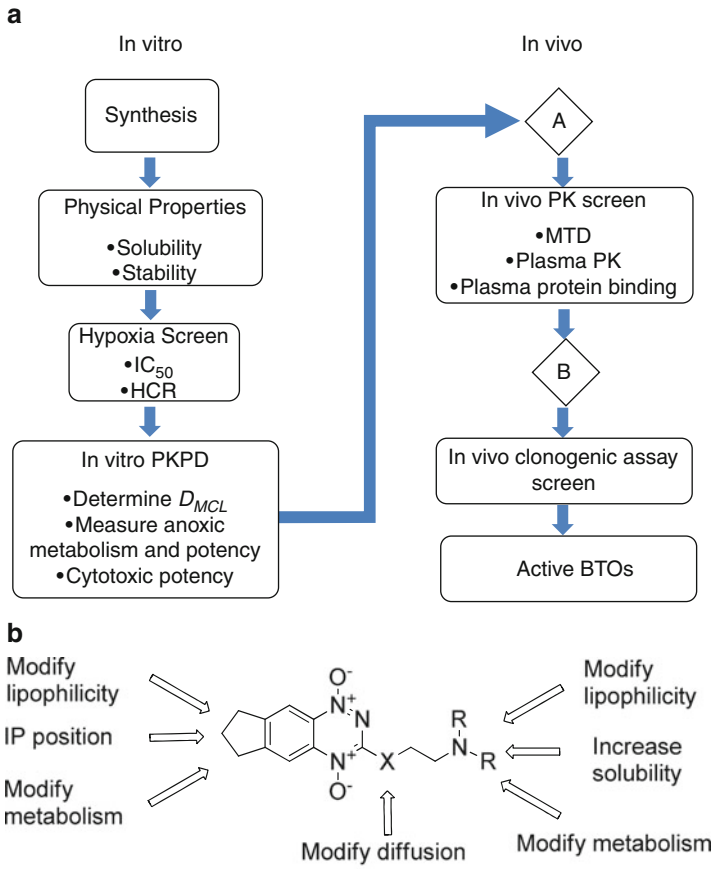

Fig. 6.7 (**a**) A pharmacokinetic/pharmacodynamic (PKPD)-guided screening algorithm that incorporates drug penetration. After initial screening for physicochemical properties and hypoxia selectivity, parameters governing drug penetration (diffusion coefficient and rate of bioreductive metabolism) were measured in vitro or calculated and used in a spatially resolved PKPD model to calculate the drug exposure (AUC) required for 1 log of cell killing in addition to radiation alone. Compounds that demonstrated in vivo hypoxia selectivity at achievable AUC (*Prediction A*) were advanced to in vivo screening (*MTD*, *plasma PK*). The model was then run with measured plasma pharmacokinetics as input, and compounds predicted to add >0.3 log cell killing in addition to radiation alone (*Prediction B*) were advanced to in vivo clonogenic assay screens. (**b**) General structure of tricyclic benzotriazine dioxides, indicating drug design considerations

to specifically consider EVT at an early stage in the drug design process and to predict in vivo hypoxia selectivity resulting from changes in EVT. Increased bioreduction produces competing effects of increasing potency and decreasing EVT; thus designing improved analogs requires optimizing potency and EVT rather than simply

maximizing any individual parameter. A range of structural variations were explored in an effort to optimize these parameters (Fig. 6.7b) (Hay et al. 2007a, b, 2008). The confidence gained using the spatially resolved pharmacokinetic/pharmacodynamic validation allowed us to screen a large number of analogs in vitro and base our SAR on predicted in vivo hypoxic cell killing rather than conduct extensive in vivo testing. Diffusion coefficients and rates of reductive metabolism in the analog series varied by more than 100-fold (Hicks et al. 2010), and a high correlation between predicted and observed activity was found in initial HT29 xenograft screening. The addition of a third saturated ring to the benzotriazine core provided reduced hypoxic metabolism and increased lipophilicity, which increased EVT and created chemical novelty to substantiate an intellectual property position. The addition of a basic amine side chain increased aqueous solubility but reduced lipophilicity and affected hypoxic metabolism, reducing EVT. While the optimization of two SARs for EVT and metabolism provided analogs with superior in vivo hypoxic selectivity, their in vivo activity was influenced by a third SAR for host toxicity. This is exemplified by the variation in maximum tolerated doses, and consequently AUC, as a function of lipophilicity and amine pKa (Fig. 6.8). Whereas SN29143 was predicted to have substantially improved activity compared to TPZ, poor plasma AUC precluded in vivo activity. Attempts to improve the pharmacokinetics by modulating lipophilicity and amine pKa led to a high AUC and improved EVT but very low hypoxic potency, which compromises the activity of SN29434. Increasing both lipophilicity and pKa increased host toxicity and lowered AUC (SN29467). Replacing the strongly electron-donating 3-amino substituent with a weaker 3-alkyl substituent led to increased EVT from increased lipophilicity and increased hypoxic potency from higher rates of metabolism (SN30000). Substituents resulting in higher lipophilicity (SN30124) and higher pKa (SN30080, SN30081) resulted in a similar trend of increasing toxicity and poorer plasma AUC, as described above. SN30000 was predicted to be substantially more active than TPZ and SN29434 as a result of low toxicity and good plasma AUC, and this was demonstrated in the HT29 xenograft model.

SN30000 emerged as the lead tricyclic BTO from this program, with broadly improved activity relative to TPZ. Aqueous solubility is improved by almost an order of magnitude (Hicks et al. 2010). SN30000 demonstrates higher potency and hypoxic selectivity than TPZ in IC_{50} assays across a panel of cell lines and in clonogenic assay in HT29 cells (Hicks et al. 2010). It is important to note that the measured K-value of SN30000 is not significantly different (1.14 ± 0.24 μM oxygen) from TPZ (1.21 ± 0.09 μM oxygen), indicating retention of the desirable property of activation at intermediate oxygen concentrations. Improved EVT for SN30000 was confirmed experimentally, with a threefold higher diffusion coefficient than TPZ in HT29 and SiHa MCLs (Hicks et al. 2010). SN30000 shows increased activity relative to TPZ against hypoxic cells in combination with single-dose or fractionated radiation in several tumor xenografts (HT29, SiHa, H460) by in vivo clonogenic assay and superior activity in SiHa xenografts with fractionated radiation by a delay in tumor regrowth (Hicks et al. 2010). SN30000 is currently in preclinical development with Cancer Research UK.

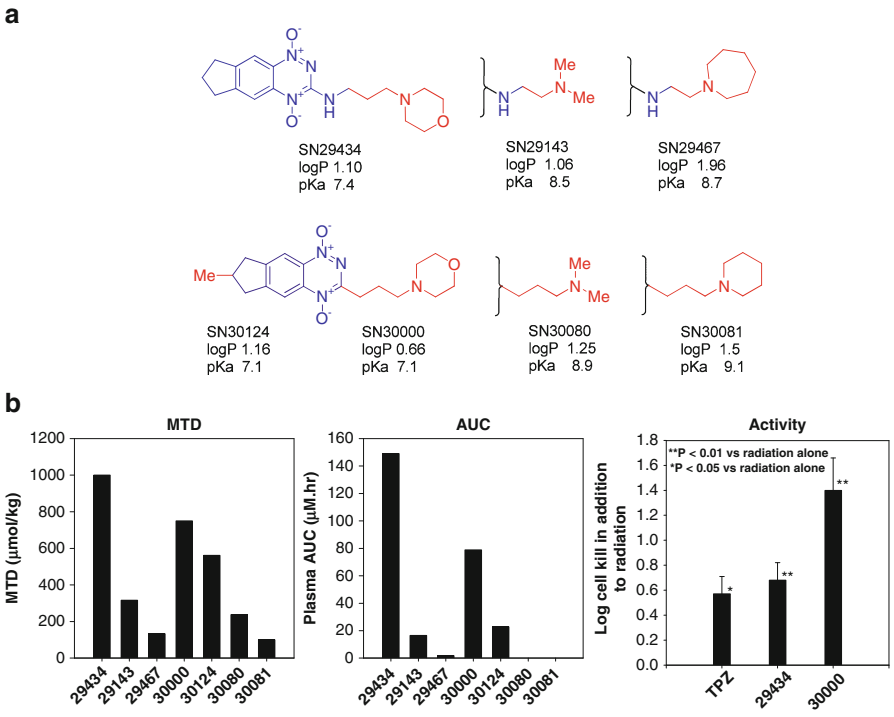

Fig. 6.8 (**a**) Structure of an amine series of tricyclic benzotriazine dioxides, indicating the range of physicochemical parameters explored. (**b**) The maximum tolerated doses (*MTDs*), drug exposure (*AUC*), and hypoxic cell killing by compounds in combination with radiation (20 Gy) in HT29 xenografts by in vivo clonogenic survival assay

6.4 Targeting the Hypoxia Response Pathway

6.4.1 Introduction

The HIF family of transcription factors is well established as the key mediator of the adaptive response to hypoxia, and their role in cancer has been extensively described (Poon et al. 2009; Semenza 2003, 2010). These transcription factors are the primary oxygen sensors and use oxygen and 2-ketoglutarate as substrates for the hydroxylation of specific proline residues on HIF-1α or HIF-2α by prolyl hydroxylase domain enzymes. This allows binding by the von Hippel-Lindau (VHL) factor and recruitment of an ubiquitin ligase complex that initiates ubiquitination and proteasomal degradation. In the absence of oxygen, HIF-1α is able to bind the constitutively expressed HIF-1β and coactivation partners, bind to hypoxia response elements (HREs), and activate transcription of a variety of genes involved in

angiogenesis, metabolic adaption, cell survival, and metastasis. However, HIF-1α activation may also be induced by other stimuli, including genetic changes to tumor suppressors (e.g., VHL [Kaelin 2008]) or tumor activators (e.g., Ras [Mazure et al. 1996]), growth factor stimulation (e.g., IGF-R [Ren et al. 2010]), and depletion of ascorbate (Kuiper et al. 2010). In addition, the differential expression and roles of HIF-1α and HIF-2α need to be considered (Carroll and Ashcroft 2006).

Inhibition of HIF-1α activity has been shown to slow angiogenesis and tumor growth in xenograft models (Maxwell et al. 1997), whereas inhibition of HIF-1α activity sensitizes hypoxic cells to conventional therapies (Moeller et al. 2004, 2005; Williams et al. 2005). The negative impact of HIF1α overexpression on treatment response and outcomes across a range of human tumors is also well described (Jubb et al. 2010; Semenza 2007). Multiple targets within the HIF-1α signaling pathway have been identified as a candidate drug targets (Giaccia et al. 2003; Semenza 2007). As a consequence, there has been a plethora of HIF-1 inhibitors that have been extensively reviewed (Poon et al. 2009; Semenza 2007; Xia et al. 2012). These inhibitors may be characterized as direct (interference with HIF-1α synthesis, stability, or binding to transcription partners and HREs) or indirect via the myriad of upstream or downstream participants in the hypoxia response network.

6.4.2 Direct HIF-1α Inhibitors

Direct inhibition of HIF1α translation has been demonstrated by a wide range of agents through multiple mechanisms, with the topoisomerase-I inhibitor topotecan the best-described example. Topotecan was identified as an inhibitor of HIF-1α translation (Rapisarda et al. 2002) by a topoisomerase-I-dependent mechanism, but at concentrations below those necessary for DNA damage–mediated cytotoxicity (Rapisarda et al. 2004a). As well as inhibiting HIF1α protein expression and tumor growth in a glioma xenograft model (Rapisarda et al. 2004b), combination of daily low-dose topotecan with bevacizumab provided significantly increased tumor cell killing in U251-HRE xenografts compared to either agent alone (Rapisarda et al. 2009). Topotecan recently completed a phase I clinical trial exploring its effect on HIF-1α, and reduced HIF-1α expression was observed in some patients (Kummar et al. 2011). CPT-11 (EZN-2208), a more potent, soluble prodrug (Sapra et al. 2008), provides improved suppression of HIF-1α and downstream gene targets (Sapra et al. 2011) and is in a phase II trial as both a cytotoxin and an HIF-1α inhibitor in combination with bevacizumab (www.ClinicalTrials.gov identifier NCT01251926).

Many of the compounds reported as HIF-1α inhibitors are not specific for HIF-1α or have multiple mechanism of action. Examples of this are seen with the HSP90 inhibitors geldanamycin and 17AAG (Isaacs et al. 2002; Mabjeesh et al. 2002) and with inhibitors of thioredoxin-1, such as PX12, which inhibits assembly of the transcription complex but has other effects (Welsh et al. 2003). PX12 has completed a phase I clinical trial in which stable disease was seen in patients with elevated levels of thioredoxin-1 (Ramanathan et al. 2007).

6.4.3 Indirect HIF Inhibitors

Indirect approaches take advantage of the network of upstream stimulating factors (e.g., the phosphoinositide 3-kinase/AKT/mammalian target of rapamycin pathway [Zhong et al. 2000] and the Ras/mitogen-activated pharmacokinetics pathway [Berra et al. 2000]) and downstream target genes (Semenza 2010) and may provide HIF-1α inhibition via multiple pathway interactions. The use of the multikinase inhibitor sorafenib in the treatment of advanced renal cell carcinoma (RCC) highlights this approach (Rini 2010). Advanced RCC is driven by HIF stabilization via the loss of functional VHL in a majority of cases and displays a highly angiogenic and invasive phenotype. Although sorafenib is primarily aimed at targeting downstream kinases involved directly in angiogenesis (vascular endothelial growth factor receptor-2 and -3 and platelet-derived growth factor receptor), its inhibition of upstream BRAF can also affect HIF-1 activity (Wilhelm et al. 2008).

6.4.4 Targeting Glucose Metabolism

Many of these downstream HIF targets are associated with the cellular reprogramming of metabolism from oxidative phosphorylation to aerobic glycolysis. This shift supports biosynthesis to maintain expansive tumor growth and presents a wide range of potential targets to disrupt tumor cell metabolism (Jones and Schulze 2012). Although regulated by other signaling factors such as p53 and Myc, HIF-1α plays an important role in the regulation of the glycolytic pathway (Cairns et al. 2011). Hypoxic cells are particularly vulnerable to reductions in the production of adenosine triphosphate, and so inhibition of glycolysis is potentially an effective strategy against hypoxic cells (Kurtoglu et al. 2007). This was first demonstrated for 2-deoxy-D-glucose (Song et al. 1976), which, after phosphorylation, inhibits hexokinases and their association with mitochondria. Although tolerated by patients in phase I/II trials, there is a dearth of published information on the efficacy of 2-deoxy-D-glucose in patients (Jones and Schulze 2012).

The glucose transporter GLUT-1 has been shown to be elevated in many tumor types and is a negative prognostic factor (Macheda et al. 2005). Although a variety of glucose transport inhibitors have been reported, many are not selective for GLUT-1 or have multiple mechanisms of action, making assessment of their value for targeting tumor metabolism difficult. For example, phloretin, a competitive inhibitor of GLUT-1, slows tumor growth (Kobori et al. 1997) and can sensitize tumor cells to chemotherapeutics under hypoxic conditions (Cao et al. 2007). However, it can also interact with the monocarboxylate lactate transporter MCT-4 (Dimmer et al. 2000).

A new strategy to identify agents that are selectively cytotoxic to cells overexpressing HIF-1α used a synthetic lethality approach (Kaelin 2005) based on VHL-deficient RCCs (Chan and Giaccia 2008; Sutphin et al. 2007). In this cell line, loss of functional VHL leads to constitutive expression of HIF-1α and mimics chronic

hypoxia. A high-throughput screen of small molecules with paired VHL-proficient/-deficient cell lines was used to identify compounds that selectively kill VHL-deficient cells (Sutphin et al. 2007). This approach furnished a series of compounds with diverse properties (Bonnet et al. 2011; Hay et al. 2010; Turcotte et al. 2008) (see Chap. 9). We conducted an SAR study around one class (3-pyridyl benzamidophenyl sulfonamides) and identified analogs with submicromolar cytotoxic potency and selectivity for von Hippel-Lindau negative (VHL-ve) RCC cells in excess of 100-fold in vitro (e.g., SN30408, also known as STF-31) (Sutphin et al. 2011). The presence of a 3-pyridyl carboxamide was key to this activity. Substituents on this ring or the central phenyl ring reduced activity. The methyl sulfonamide linker was required for activity, whereas a wide range of substituents were tolerated on the terminal ring. This SAR was used to design affinity chromatography reagents that selectively bind to GLUT-1 (Chan et al. 2011). Molecular modeling studies using a homology model of GLUT-1 (Salas-Burgos et al. 2004) predicted that SN30408 and related molecules could bind within the central solute channel and interact with ARG126 and TRP412, both key residues for glucose transport (Brockmann et al. 2001). SN30408 seems to occupy a similar binding location to fasentin (Wood et al. 2008) and a series of recently described thiazolidinedione inhibitors that inhibited glucose transport in LNCaP prostate carcinoma cells (Wang et al. 2012a). SN30408 was shown to bind to GLUT-1 and selectively inhibit glucose uptake into VHL-ve RCC cells that overexpress GLUT-1, resulting in necrotic cell death (Chan et al. 2011). A key concern with targeting glucose transport is the effect on normal tissues, such as in the case of GLUT-1, erythrocytes, and the blood-brain barrier. Although it reduced glucose uptake into erythrocytes, SN30408 did not cause hemolysis. This was further monitored in vivo, where ^{18}F-2-fluorodeoxyglucose positron-emission tomography (PET) demonstrated that VHL-ve tumors had high uptake of glucose and that treatment with nontoxic doses of a more soluble analog (SN31154) consistently reduced this uptake while having a minimal effect on the use of glucose in the brain. Daily treatment with a nontoxic dose of SN31154 over 14 days inhibited tumor growth in vivo (Chan et al. 2011).

6.5 Identifying the Target in Patients

As targeted therapies move into the clinic, it becomes increasingly important to identify patients with susceptible tumor cell populations who may benefit clinically (Basu 2010; Mok 2011). To fully exploit hypoxia with targeted therapy, the use of biomarkers to select suitable patients and assess response to treatment will greatly aid clinical development.

While polarographic electrodes have demonstrated a wide range of oxygen tensions in solid tumors (Nordsmark et al. 2005) and hypoxia status has been related to outcome in a range of tumor types (Vaupel and Mayer 2007), this approach is limited to accessible tumors. Tumor oxygenation may also be evaluated using nuclear

magnetic resonance techniques with exogenous fluorocarbon markers for ^{19}F nuclear magnetic resonance or blood oxygen level–dependent magnetic resonance imaging (Tatum et al. 2006) (See Chap. 16).

Recent reports of hypoxic gene signatures in various cancer sites (Buffa et al. 2010; Chi et al. 2006; Jubb et al. 2010; Murat et al. 2009; Winter et al. 2007) have related clinical outcome following standard treatments. A signature of 15 hypoxic genes was developed, validated, and used to retrospectively analyze head and neck squamous cell carcinoma (HNSCC) samples from the DAHANCA5 trial (Toustrup et al. 2011). This analysis demonstrated that only patients with hypoxic tumors defined by the hypoxic gene signature benefited from nimorazole.

Exogenous nitroimidazole hypoxia probes such as pimonizadole or EF5, with immunostaining by antibodies to the reduced adducts (Evans et al. 2000; Raleigh et al. 1998), have been used clinically. In a substudy of the ARCON trial pimonidazole was used to measure tumor hypoxia in patients with laryngeal cancer. For patients with higher pimonidazole labeling, ARCON provided benefit in terms of local control and 5 years of disease-free survival (Janssens et al. 2012).

More convenient approaches using circulating surrogate hypoxic markers in blood, such as osteopontin (Le et al. 2003), hepatocyte growth factor, and interleukin-8 (Le et al. 2012) have provided equivocal results. In the DAHANCA5 trial, patients with high levels of plasma osteopontin were shown to benefit from the addition of nimorazole, while patients with intermediate or low osteopontin showed no benefit (Overgaard et al. 2005). However, osteopontin failed to show any correlation with adverse outcome or benefit from the addition of hypoxia-targeted therapy in the TROG 02.02 phase III trial, in which patients with stage III/IV HNSCC received chemoradiotherapy and TPZ (Lim et al. 2012). In the same trial, two other hypoxic markers – hepatocyte growth factor and interleukin-8 – gave some predictive indication (Le et al. 2012).

PET using 2-nitroimidazole–based markers such as ^{18}F-misonidazole (Lee et al. 2009), ^{18}F-EF5 (Koch et al. 2010; Komar et al. 2008), and ^{18}F-HX4 (Dubois et al. 2011; van Loon et al. 2010) has been explored as a noninvasive method for measuring hypoxia (Horsman et al. 2012). In a phase II trial of patients with HNSCC who were treated with chemoradiotherapy with or without TPZ, patients with hypoxic tumors identified using ^{18}F-fluoromisonidazole PET fared significantly better when treated with TPZ compared to standard chemoradiotherapy (Rischin et al. 2006). Despite this, PET was not used for patient selection in the subsequent phase III trial, which failed to demonstrate a benefit for the addition of TPZ to chemoradiotherapy (Ang 2010; Rischin et al. 2010b).

The clinical development of HAPs would benefit from biomarkers that interrogate multiple elements of their sensitivity. We recently demonstrated that the hypoxic activation of EF5 is highly correlated with activation of SN30000 (and TPZ) across a panel of human tumor cell lines (Wang et al. 2012b). This study suggests that PET imaging with [^{18}F]-EF5 will report on both hypoxia and the activity of the one-electron reductases for SN30000 in hypoxic regions of tumors, without having to identify all the contributors to activation.

6.6 Conclusions

Although there is clear evidence that hypoxia limits the response to therapy, extensive drug discovery efforts have delivered limited success in clinically targeting hypoxia. This failure may be attributed in part to difficulties faced by academic groups and small biotechnology companies advancing novel agents to clinical trial. It is beneficial to develop agents in combination with radiotherapy when hypoxia contributes greatly to resistance to therapy. An important issue is the failure to recognize hypoxia-directed drugs as targeted therapies, develop biomarkers to aid in the selection of patients for treatment, and monitor response. In each of three hypoxia-directed approaches under development in our laboratories, we are identifying appropriate biomarkers, while the radiosensitizer and SN30000 will be developed in conjunction with radiotherapy.

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