Bioreductive prodrugs as cancer therapeutics: targeting tumor hypoxia

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Abstract

Hypoxia, a state of low oxygen, is a common feature of solid tumors and is associated with disease progression as well as resistance to radiotherapy and certain chemotherapeutic drugs. Hypoxic regions in tumors, therefore, represent attractive targets for cancer therapy. To date, five distinct classes of bioreactive prodrugs have been developed to target hypoxic cells in solid tumors. These hypoxia-activated prodrugs, including nitro compounds, N-oxides, quinones, and metal complexes, generally share a common mechanism of activation whereby they are reduced by intracellular oxidoreductases in an oxygen-sensitive manner to form cytotoxins. Several examples including PR-104, TH-302, and EO9 are currently undergoing phase II and phase III clinical evaluation. In this review, we discuss the nature of tumor hypoxia as a therapeutic target, focusing on the development of bioreductive prodrugs. We also describe the current knowledge of how each prodrug class is activated and detail the clinical progress of leading examples.

Key words Bioreductive, prodrug, tumor hypoxia, clinical trial, oxidoreductase

Hypoxia plays a central role in cancer progression. Indeed, tumor hypoxia can promote resistance to apoptosis[1,2], encourage hypermutation by inhibiting DNA repair[3], alter cell metabolism to favor cell growth[4,5], up-regulate angiogenesis[6], enhance local invasiveness[7], drive metastatic spread[8], and provide a sanctuary for cancer stem cells[9]. This plethora of effects on tumor biology is orchestrated in large part by the oxygen labile transcriptional regulator, hypoxia-inducible factor 1[10].

Tumor hypoxia also plays an important role in resistance to radiotherapy and chemotherapy[11]. Molecular oxygen is a potent radiosensitizer, as it facilitates oxidation of free radicals in DNA generated during tissue irradiation. Accordingly, hypoxic cells in tumors are directly and significantly resistant to radiotherapy[12,13]. In addition, hypoxic cells can exhibit considerable resistance to

chemotherapy via several mechanisms. For example, hypoxic cells—often quiescent because of the lack of oxygen and nutrients—can escape the actions of chemotherapeutic drugs with anti-proliferative properties, such as anti-metabolites[14]. These S-phase–specific agents are incorporated into the DNA of dividing cells, a process necessary for their activity. Resistance may also be due to the inherent limitations of delivering chemotherapy to distal tumor regions; hypoxic cells reside in a pharmacological sanctuary[15,16].

With the increasing global incidence of cancer, efficient and specific strategies for cancer treatment are urgently required. The hypoxic microenvironment of solid tumors has attracted significant attention as a target for the development of a novel therapeutics for cancer treatment. Bioreductive prodrugs can be designed for selective activation under low oxygen conditions typical of many solid tumors. These hypoxia-activated prodrugs can target and kill hypoxic cells, and their effect can extend to include sterilization of surrounding tumor cells when the activated metabolites are sufficiently stable to diffuse beyond their primary site of action. The majority of normal tissues are devoid of hypoxic region, although several tissues can exhibit regions of mild physiological hypoxia. Therefore, the severity of hypoxia observed in many solid tumors represents an attractive basis for tumor selectivity. In this article, we aim to highlight the various classes of bioreductive prodrugs and their metabolism by endogenous oxidoreductases. We also discuss the lessons learnt from past drug design and the future of this field.

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Mechanisms of Bioreductive Prodrug Activation

Hypoxia-activated prodrugs are deactivated or masked cytotoxins that undergo biotransformation following reductive metabolism by endogenous human cellular oxidoreductases. This process is usually inhibited by molecular oxygen, thereby imparting specificity for the hypoxic tumor microenvironment. Oxygen inhibition involves direct competition for the single electron of the initial reduced drug species, and direct scavenging of this single electron by oxygen prevents net reduction of the prodrug. The superoxide radical byproduct of this process is readily detoxified by superoxide dismutase, ensuring bioreductive drugs exhibit minimal toxicity to normal tissues[11,17].

This activation step is catalyzed by a variety of oxidoreductases and differs depending on the bioreductive drug class[18]. In the majority of cases, this reduction is inhibited in the presence of oxygen. Preclinical models have increased our understanding of the enzymes involved in bioreductive metabolism, but further studies are needed. In addition, few studies have examined, in the clinical setting, the roles these enzymes play.

One-electron and two-electron oxidoreductases typically catalyze oxygen-sensitive and oxygen-insensitive activation of bioreductive prodrugs, respectively. One-electron oxidoreductases generate prodrug free radical species that are readily back-oxidized resulting in a futile metabolic cycle[19]. This reversible step ensures prodrug activation is restricted to tissues experiencing limited oxygen availability. In contrast, two-electron reduction by certain oxidoreductases fails to generate an oxygen-sensitive radical intermediate. This metabolic process is therefore irreversible and may occur in tumors and normal tissues. In some cases, this can result in oxygen-independent prodrug activation.

Although a variety of one-electron or two-electron oxidoreductases are known to be involved in prodrug reduction, the frequency and amplitude of their expression in human tumors is poorly defined. To date, we have shown that the diflavin oxidoreductases, such as cytochrome P450 oxidoreductase (POR), activate bioreductive prodrugs in human cell cultures; however, the frequency of expression in human cancers appears to be low and generally does not overlap with biological markers of hypoxia such as carbonic anhydrase IX[20]. Several two-electron oxidoreductases such as DT-diaphorase (NQO1) and aldo-keto reductase 1C3 (AKR1C3) are expressed in certain tumor types, but NQO1 and AKR1C3 have also been found in normal tissues.

Progress in the Development of Hypoxia-Activated Prodrugs

Five classes of bioreductive compounds that can undergo enzymatic reduction to active species have been developed. These can be divided into nitro(hetero)cyclic compounds, aromatic N-oxides, aliphatic N-oxides, quinones, and metal complexes (for example structures, as shown in **Figure 1**). Although no registered agents have been used in clinical therapy, several hypoxia-specific prodrugs are in various stages of clinical development.

Nitro(hetero)cyclic compounds

Several nitroaromatic compounds have been evaluated in clinical trials, including the nitroimidazoles misonidazole, etanidazole, and nimorazole. These agents were primarily designed as radiosensitizers (i.e., oxygen mimetics), and derivatives have been developed for hypoxic cell imaging using immunohistochemistry [e.g., pimonidazole, EF5 [2-(2-Nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl) acetamide] or positron emission tomography (PET; e.g., [^{18}F]-floromisonidazole, [^{18}F]-EF5, [^{18}F]-flortanidazole). More recently, the transition in electron density resulting from reduction of a nitro group (NO_2) to a hydroxylamine (NHOH) or amine (NH_2) has been utilized to design the hypoxic cytotoxin PR-104.

PR-104 is a water-soluble phosphate ester pre-prodrug that undergoes rapid hydrolysis *in vivo* to the prodrug PR-104A[21]. PR-104A is a dinitrobenzamide mustard that can be reduced to *para*-hydroxylamine and para-amine metabolites—PR-104H and PR-104M, respectively—by various oxidoreductases. These cytotoxic metabolites give rise to DNA interstrand cross-links, which can kill tumor cells[22]. Flavoenzymes mediate the nitro reduction of PR-104A under anoxia *in vitro*, with the one-electron reductase POR appearing to account for the majority of activity in human tumor cell lines[20,23]. In addition, the two-electron oxidoreductase AKR1C3 can also catalyze the reduction of PR-104A and has been identified as an oxygen-insensitive metabolic pathway[24]. The anti-cancer agent PR-104 is currently in phase II clinical trials.

Another promising clinical stage nitro compound is TH-302, a 2-nitroimidazole-based nitrogen mustard prodrug. Reduction by one-electron oxidoreductases in hypoxic cells leads to fragmentation of the 2-nitroimidazole trigger unit and release of bromo-isophosphoramide mustard[25]. The mustard moiety acts as a DNA cross-linking agent and appears particularly effective in cell lines deficient in homologous recombination DNA repair pathways[26]. TH-302 shows good selectivity for hypoxic cells, with reported aerobic-to-hypoxic cytotoxicity ratios *in vitro* of up to 550[26]. Efficacy *in vitro* was translated to efficacy in preclinical *in vivo* studies[27,28] and enabled TH-302 to progress to clinical trial. The reported phase I trial demonstrated encouraging evidence of tumor response in a monotherapy setting[29]. TH-302 in combination with chemotherapy is currently being evaluated in several phase II and phase III clinical trials.

Aromatic N-oxides

The N-oxide tirapazamine (TPZ; SR4233) has been the most extensively evaluated compound in the clinic to date. TPZ was first reported in 1986 and was shown to exhibit up to 300-fold greater toxicity under anoxic conditions than aerobic conditions *in vitro*[30]. TPZ undergoes one-electron reduction to generate a TPZ radical; in the absence of oxygen, the TPZ radical undergoes spontaneous conversion to generate the benzotriazinyl radical, leading to DNA breaks and other complex lesions[31-33]. When oxygen is present, TPZ undergoes futile cycling back to the parent compound with the concomitant formation of superoxide. A number of enzymes
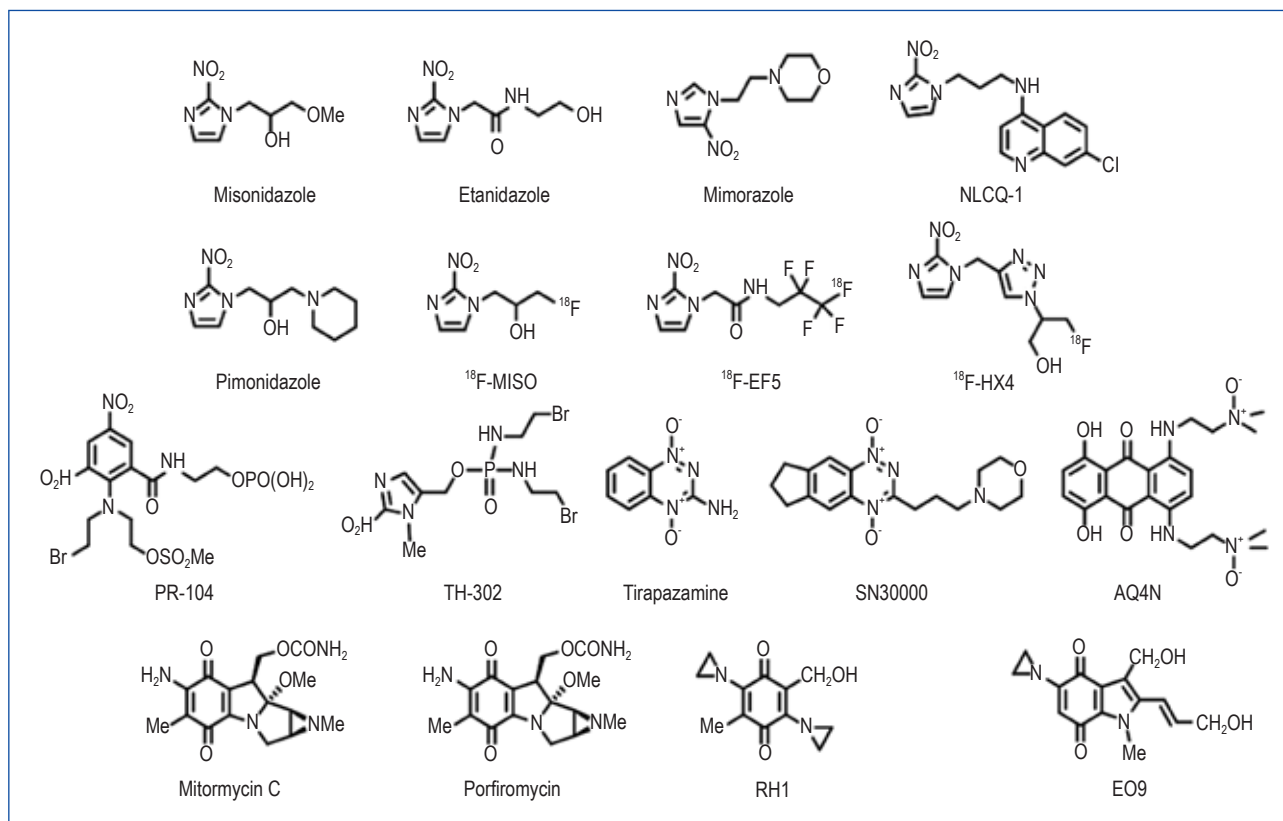
Figure 1. **Structure of the bioreductive compounds.**

catalyze the one-electron reduction of TPZ[34], with POR being the most extensively reported in the literature[35,36]. In addition to the one-electron reduction of TPZ, two-electron reduction has also been reported by enzymes such as NQO1[34]. Two-electron reduction of TPZ is bioprotective to cells because it bypasses formation of the TPZ radical to generate the mono N-oxide, a relatively non-toxic metabolite[37].

Preclinical *in vivo* studies in which TPZ was combined with radiotherapy or cisplatin[30,38,39] showed great promise, and TPZ progressed to clinical trials in the early 1990s. TPZ has been evaluated in a number of phase II trials, with promising results reported in most trials[40-43]. However, phase II results have not been translated into increased efficacy over conventional treatment in phase III trials[44,45]. The failure of TPZ in phase III trials may reflect the need to identify patient populations with high levels of tumor hypoxia and thus allow TPZ to be administered to patients most likely to benefit from the drug.

SN30000 is an analogue of TPZ that has undergone extensive optimization of its diffusion and metabolism characteristics[46]. This allows the prodrug to reach the hypoxic tumor cell compartment in higher concentrations than TPZ. Consequently, hypoxic radiation-resistant tumor cells are more effectively sterilized[46]. SN30000 is presently scheduled to enter phase I clinical trials.

Aliphatic N-oxides

The leading aliphatic N-oxide AQ4N (banoxantrone) is metabolized under hypoxia to AQ4, a high affinity DNA intercalator that inhibits topoisomerase II[47]. Unlike aromatic N-oxides, oxygen-sensitive reduction of AQ4N involves a two-electron step carried out by cytochrome P450 isozymes (CYP)[48-52] or inducible nitric oxide synthase (NOS2A)[53]. Selectivity for hypoxic conditions occurs because this enzymatic step is inhibited in the presence of oxygen. Preclinical studies combining AQ4N with radiation or chemotherapy *in vivo* demonstrated significant activity, enabling AQ4N to advance to clinical trials[54,55]. Metabolism of AQ4N to AQ4 in tumor tissue has been demonstrated in clinical studies[56].

Quinones

The development of quinones as bioreductive drugs stems from an observation made in 1980 that the quinone mitomycin C (MMC) is preferentially activated in hypoxic tumor cells[57]. However, although MMC is preferentially activated in hypoxic cells, this effect is minor, prompting development of other quinone compounds that show greater selectivity towards hypoxic cells, including porfiromycin[58], RH1[59], and EO9 (apaziquone)[60]. Activation of quinones under hypoxia is carried out by one-electron reductases such as POR[61,62].

The greatest selectivity towards hypoxic cells has been observed for the indolequinone EO9[63-65]. Hypoxic selectivity is lost in cells expressing the two-electron oxidoreductase NQO1[66,67]. One of the drawbacks of EO9 is poor pharmacokinetic property. Thus, EO9 has been evaluated in a phase II trial in bladder cancer, where loco-regional administration of the drug is possible[68]. In addition, reported expression of NQO1 in a subset of bladder cancer patients ensures activation of EO9 in this setting[69]. EO9 is currently being evaluated against bladder cancer in phase III trials.

Metal complexes

Complexes of transition metals have the potential to be used as hypoxia-selective agents, but to date, none have been developed for clinical use. The first record of metal complexes as hypoxia-selective agents was in 1993, when a series of nitrogen mustard-cobalt complexes were developed[70]. The rationale behind this class of compound is that cytotoxicity depends on the electron density on the nitrogen mustard. Coordination of the nitrogen lone pair of electrons to Co(III) suppresses the alkylating reactivity. Under hypoxic conditions, one-electron reduction of Co(III) to Co(II) can occur and lead to an increase in mustard reactivity[70]. More recently, hypoxia-selective complexes of cobalt/chloromethylbenzindoline DNA minor groove alkylators[71] and copper/nitrogen mustards[72] have been reported.

Summary and Future Perspectives

Hypoxia, a common phenomenon of solid tumors, is a unique physiological feature of cancer that can be exploited with rational drug design. To this end, a number of bioreductive prodrugs have been designed in the last three decades. Although several hypoxia-selective prodrugs have progressed to clinical trials, none have yet been approved for clinical use. This partly reflects some of the many challenges and limitations that have come to light during the development of these agents. For example, TPZ, the most advanced clinical candidate, suffers from excessive metabolic consumption as it penetrates the extravascular space, a phenomenon that diminishes its apparently impressive selectivity for hypoxic cells from 50–300 fold *in vitro* to the more modest range of 3–5 fold *in vivo*[73,74]. EO9 encountered similar issues of poor extravascular transport, compounded by an extraordinarily brief plasma half-life and oxygen-independent metabolism by NQO1[60]. Metabolic reduction by concerted two-electron oxidoreductases can unexpectedly corrupt the

oxygen-inhibited activation as recently reported for PR-104[24]. Here, AKR1C3 bypasses the desired mechanism of one-electron reduction, rendering all AKR1C3-positive tissues potentially susceptible to PR-104 toxicity, including the gastrointestinal tract and bone marrow. An interesting exception is AQ4N, for which concerted two-electron reduction is inhibited by molecular oxygen, presumably via direct competition in the active site of the cytochrome P450 isozymes. Beyond these idiosyncratic examples of hypoxia prodrug design challenges lies a fundamental need to balance small molecule stability against reactivity in a reduction/oxidation equilibrium, such that toxicity is only "delivered" to tissues that oxygen is unable to reach. One example where this harmony has apparently been achieved is TH-302[26]. Indeed, early reports of the anti-tumor efficacy of TH-302 in clinical trials garner optimism[29,75,76]. Most recently, the departure from DNA damaging chemistries to a new generation of prodrugs that release kinase inhibitors under hypoxia may signal an exciting new direction in prodrug design[77]. The knowledge gained from preclinical and clinical studies, as well as an increased understanding of cancer biology, can assist in the rational development of novel drugs or drug analogues. This may lead to more efficient targeting of the hypoxic tumor environment in the future. It is likely that the future will see a move towards a more personalized medicine approach, with the identification of patients that may benefit most from hypoxia-selective drugs. Achieving this aim will involve the use of imaging agents to detect hypoxia in the clinic, thus enabling hypoxia-selective prodrugs to be directed towards the patients most likely to benefit most from treatment. In addition, developing a thorough understanding of the enzymology of bioreductive drugs is an important step in identifying tumor types that express high levels of the activating enzymes and enables targeting to these tumor types. Whether prodrug activation in a clinical setting depends on a limited number of key enzymes or whether it occurs from activation of a wide variety of enzymes remains to be seen.

Acknowledgment

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