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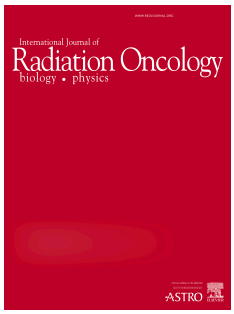

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Clinical advances of hypoxia-activated prodrugs in combination with radiotherapy

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Abstract

With the increasing incidence of cancer worldwide, the need for specific, effective therapies is ever more urgent. One example of targeted cancer therapeutics is hypoxia-activated prodrugs (HAPs), also known as bioreductive prodrugs. These prodrugs are inactive in cells with normal oxygen levels but in hypoxic cells (with low oxygen levels) undergo chemical reduction to the active compound. Hypoxia is a common feature of solid tumors and is associated with a more aggressive phenotype and resistance to all modes of therapy. Therefore, the combination of radiotherapy and bioreductive drugs presents an attractive opportunity for synergistic effects, as the HAP targets the radiation resistant hypoxic cells. HAPs have typically been precursors of DNA damaging agents, but a new generation of molecularly targeted HAPs is emerging. By targeting proteins associated with tumorigenesis and survival, these compounds may result in greater selectivity over healthy tissue. We review the clinical progress of HAPs as adjuncts to radiotherapy, and conclude that the use of HAPs alongside radiation is vastly underexplored at the clinical level.

Introduction

Regions of low oxygen (hypoxia) arising from an imbalance in cellular oxygen demand and availability are a common feature of solid tumors. Both diffusion and perfusion limitations contribute to the prevalence of hypoxia in solid tumors. Diffusion limitations arise largely as a result of insufficient vasculogenesis during tumor growth, and these insufficiencies are confounded by the structural and functional abnormalities of the tumor microvasculature [1]. This heterogeneity is conducive to erratic blood flow and temporary occlusions, such that acute perfusion limitations cause cycling hypoxia and reoxygenation. Hypoxia drives cancer progression and is a negative prognostic and predictive factor, as oxygen-deprivation promotes tumor characteristics that contribute to a more clinically aggressive, treatment-resistant phenotype [2]. In particular, hypoxia is associated with increased invasiveness, metastasis, genomic instability, the suppression of apoptotic signaling, and significant resistance to chemotherapy and radiotherapy [3-6] (Figure 1A). Chemoresistance in hypoxic tumors is largely a result of the inability of anti-proliferative drugs to target cancer cells that have undergone hypoxia-induced reversible quiescence [7,8]. In addition, the diffusion and perfusion limitations that cause tumor hypoxia reduce drug delivery to these regions [9-12].

The failure of radiotherapy in hypoxic tumors is primarily attributed to decreased fixation of DNA damage due to a lack of molecular oxygen [13,14]. Ionizing radiation causes cell death by inducing ionization on, or very close to, DNA and producing a radical species on the DNA. This radical can then be oxidized (predominantly by oxygen), which makes the damage permanent, or reduced (principally by thiol-containing compounds), that can restore the DNA to its original form [15]. Therefore, hypoxic tumors suffer less DNA damage, particularly DNA double strand breaks, when irradiated. Significant resistance to radiation is observed at >0.13% O_2 concentrations (radiobiological hypoxia) (Figure 1B) [16]. In addition, hypoxia increases production of vascular endothelial growth factor A (VEGFA), leading to the formation of abnormal blood vessels which can promote tumor reoccurrence following radiotherapy [17].

Current chemotherapeutics are extremely toxic and cause adverse side effects for patients. Therefore, the use of prodrugs in cancer therapy is potentially superior to conventional therapy, providing more

targeted treatment. Hypoxia describes a state of insufficient oxygen concentrations which can be present in tumors, normal tissues and wounds [16]. Normal tissue oxygen levels, often described as 'physoxia', vary from 1% to 8% oxygen with an average of around 5% [16,18]. Oxygenation in untreated tumors is significantly lower, with median oxygen levels of <2% and a range of <0.1% to 4.2% oxygen [18]. Therefore, hypoxia represents a feature of malignant tissue that is not present to the same degree in healthy tissues. With this in mind, a plethora of hypoxia activated (bioreductive) prodrugs have been designed to exploit the unique microenvironment of hypoxic tumors for personalized cancer medicine. In addition, hypoxic cytotoxins are ideal to combine with ionizing radiation as they produce a profile of toxicity as a function of distance from active blood vessels that is the opposite to that produced by ionizing radiation (Figure 1B) [19]. DNA damage, and cell killing, by radiation or conventional chemotherapeutics is generally diminished with increased distance from the blood vessel. In contrast, a drug that is preferentially cytotoxic in hypoxic cells would display the opposite trend. Therefore, HAPs in combination with radiotherapy could present a unique therapeutic combination where the two should yield complementary killing, as hypoxia-activated drugs kill the tumor cells that are resistant to ionizing radiation.

Hypoxia is highly heterogeneous, both spatially and temporally, within and between tumors [20]. Therefore it may be desirable that HAPs release stable and diffusible cytotoxins capable of killing the surrounding tumor cells at a higher oxygen concentration (which may not themselves be capable of prodrug activation) [21]. This is known as the bystander effect and has been suggested to be an important factor in the ability of HAPs to overcome the radioresistance of hypoxic tumors [21,22].

In this review, we highlight the progress in development of bioreductive prodrugs, with a focus on their use as radiosensitizers. We then discuss the existing challenges preventing their success in the clinic, and describe an emerging new generation of molecularly targeted bioreductive prodrugs that present exciting opportunities to overcome the limitations of currently available therapies.

Mechanism of reduction of HAPs

A central concept in targeting tumor hypoxia is that of bioreductive prodrugs. These agents are inactive compounds that are reduced selectively in hypoxic conditions by endogenously expressed oxidoreductases, resulting in the generation of an active anti-neoplastic effector (Figure 1C). Compounds are inactivated by the attachment of a bioreductive protecting group at a position which results in substantially reduced activity compared to the active parent compound, which is released upon reduction and fragmentation [23].

A range of chemical functionalities have been identified as useful moieties for bioreduction and these can be grouped into five main types: nitro compounds, aromatic *N*-oxides, aliphatic *N*-oxides, quinones, and transition metal complexes (Figure 2). Most commonly, reduction of these agents *in vivo* is initiated by one-electron reductases resulting in the formation of an oxygen-sensitive intermediate. Hypoxic selectivity via one-electron reduction is typically mediated by the scavenging of the received electron by oxygen, resulting in the futile redox cycling of the prodrug [24]. The superoxide by-product of this process is detoxified by superoxide dismutase, ensuring minimal toxicity to normal tissues [25,26]. In the absence of oxygen, further enzyme-mediated reduction occurs, resulting in progression to the active compound. Despite the rapid detoxification of superoxide by superoxide dismutase, it must be ensured that the cytotoxicity of the active agent exceeds that of the radicals produced in normoxic tissues [27]. A number of one electron reductases responsible for oxygen dependent prodrug activation have been identified, and their ability to do so depends on the class of bioreductive group employed [28,29].

In contrast to one-electron reduction by flavin-dependent oxidoreductases, two-electron reduction of bioreductive prodrugs generally represents an oxygen-insensitive mechanism of activation that can occur in normoxic tissues. This off-target two-electron reduction is exemplified by the reduction of the bioreductive prodrug PR-104 by human aldo-keto reductase 1C3 in normoxia [30]. Oxygen-sensitive two-electron reduction is, however, observed in prodrug activation by cytochromes p450, in which it is thought that oxygen directly competes with the target protein for the heme prosthetic group [27]. Whilst generally considered a confounding factor in the development and clinical utility of

bioreductive prodrugs, the action of endogenous two-electron reductases may be exploitable in tumors where these enzymes are significantly upregulated.

Combining HAPs with ionizing radiation

Nitro-based HAPs

Nitro-based HAP were amongst the first to be shown to undergo the oxygen-sensitive redox cycling that is characteristic of bioreductive prodrugs [31]. Early members of this class, metronidazole and misonidazole mimic the radio-sensitization caused by oxygen in normoxic tissues, in tumor cell lines and pre-clinical animal models [32,33]. Therefore, these compounds were thought to hold much promise in improving cancer therapy. However, clinical results were disappointing, and neither compound resulted in a statistically significant increase in survival compared to traditional radiotherapy alone [33-35]. An important factor in their failure was thought to be the low radio-sensitizing concentrations achievable with the tolerable dose of the drugs [33]. Attempts to improve the efficacy of nitroaryl-based HAPs in the clinic resulted in the development of pimonidazole, etanidazole, and nimorazole, bioreductive agents primarily designed as oxygen-mimetic radiosensitizers. Although pimonidazole showed no significant benefit in combination with conventional radiotherapy for the treatment of cervical carcinoma, it is widely used for hypoxic cell imaging using immunohistochemistry [36]. Etanidazole progressed to phase II and phase III clinical trials, but it too showed no positive improvement to treatment of squamous cell cancer of the head and neck (HNSCC) or small-cell lung cancer (SCLC) in combination with radiotherapy [37,38].

The success of nitroimdazoles in pre-clinical models relied on the rapid biodistribution and clearance of the agents in mice. However, the long half-lives of these compounds in humans resulted in greater toxicities, preventing the high doses necessary to result in sufficient tumor drug concentration for radiosensitization [39,40]. In addition, the cumulative toxicity of the drugs makes it difficult to combine an optimal drug schedule with fractionated radiotherapy [40].

Nimorazole, however, significantly improved the effect of radio-therapeutic management of tumors of the head and neck, without major side effects [41]. It should be noted that clinical evaluation of nimorazole in the Danish Head and Neck Cancer Study (DAHANCA 5-85) benefited from careful study design with a large cohort and tight controls, giving greater statistical power [40,41]. It is now standard of care for the treatment of HNSCC in Denmark [41,42]. Development of nimorazole in combination with radiotherapy for the treatment of HNSCC is ongoing (NCT01880359). A DAHANCA trail of hyperfractionated radiotherapy with cisplatin and nimorazole in p16 negative HNSCC was recently completed (DAHANCA 28A). Two further trials aimed at improved stratification of patients with hypoxic tumors — guided by 15-gene hypoxic signature (DAHANCA 30) [43]or FAZA-PET imaging (DAHANCA 33) — are currently, or are soon to be, recruiting patients (NCT01733823, NCT02661152, NCT02976051). Nimorazole is also being investigated in the UK in patients with HNSCC undergoing radiotherapy who are not suitable for concurrent cisplatin or cetuimab (NIMRAD, NCT01950689). This trial also involves the testing of a 26-gene hypoxic signature to predict the benefit of hypoxia modification to radiotherapy [44].

Following the initial interest in oxygen mimetics, as knowledge on the molecular mechanism and physiological changes induced by hypoxia increased, new strategies to exploit these pathways to target hypoxic cells were developed [14,45]. Oxygen-mimetic bioreductive agents were followed by a new generation of DNA targeting bioreductive agents, prodrugs activated to cytotoxic products in the hypoxic environment. Development of cytotoxic nitro compounds has culminated in PR-104 and TH-302. PR-104 is a phosphate ester pre-prodrug that undergoes hydrolysis by phosphatases to generate the prodrug PR-104A. In turn, PR-104A is reduced by one- and/or two- electron reductases to two distinct cytotoxic metabolites: PR-104H and PR-104M [30]. Both of these cytotoxins mediate cell killing through the introduction of DNA inter-strand cross-links. Following the establishment of a tolerated dose in phase I trials, PR-104 was evaluated in combination with Docetaxel in a phase II trial in SCLC (NCT00544674) [46]. However, as the trial was taking place, *in vitro* reductase profiling of PR-104 revealed that in addition to one-electron reduction, PR-104 is also activated independently of hypoxia by aldo-keto reductase 1C3 (AKR1C3) [30,47]. It emerged that SCLC does

not express meaningful levels of AKR1C3 to affect prodrug reduction, and the trial was terminated, highlighting the importance of extensive pre-clinical evaluation of prodrugs. In contrast, non-small cell lung cancer (NSCLC) has been shown to express high levels of AKR1C3, however, a trial of PR104 versus Docetaxel in NSCLC was terminated as interim analysis indicated low probability of a clinically significant result (NCT00862134). A more recent preclinical study of the efficacy of PR-104 in breast cancer tumor xenografts indicated that PR-104 (and TH-302) sensitized tumors to irradiation, particularly in BRCA2-knockout mutants. However, no clinical trials of PR-104 in combination with radiotherapy have yet been conducted.

TH-302 (Evofosafamide) is a similar compound that is reduced in hypoxic conditions to form bromo-isophosphoramide mustard (Br-IPM), a potent alkylating DNA cross-linking agent. TH-302 showed significant promise in phase II clinical trials in combination with Gemcitabine for the treatment of pancreatic cancer and in combination with doxorubicin in soft tissue sarcoma, despite increased hematologic toxicity of doxorubicin [48,49]. However, two large phase III trials have recently reported that this agent, in combination with other chemotherapeutics, was ineffective in increasing overall survival in advanced pancreatic cancer (NCT01746979) and soft tissue sarcoma (NCT01440088) [50]. TH-302 has also demonstrated activity as a radiosensitizer, specifically in hypoxic cells. In pre-clinical models of rhabodomyosarcoma (skeletal muscle) and NSCLC tumor bearing animals, TH-302 treatment resulted in tumor growth delay, which was further increased with radiotherapy [51]. The efficacy of treatment was shown to depend on tumor oxygenation (as measured by [^{18}F]HX4-PET imaging), where an increased hypoxic fraction enhanced the benefit of TH-302 [51]. In addition, in a recent study in patient-derived xenograft models of pancreatic cancer, combination of TH-302 and irradiation was more effective than either treatment alone at controlling tumor growth [52]. TH-302 specifically targeted the hypoxic zone of tumors and also induced DNA damage in tumor tissue adjacent to the hypoxic zone (bystander effect) [52]. Therefore, this drug could hold much potential for increasing the efficacy of radiotherapy in the treatment of solid tumors. However, of the 26 trials listed on the U.S National institute of Health clinical trials database, only one proposes the combination of TH-302 with radiotherapy (NCT02598687). Unfortunately, this

phase I study of TH-302 in combination with preoperative chemo-radiotherapy, for the treatment of esophageal cancer was withdrawn prior to enrolment due to the failure of the two phase III trials above to meet their primary endpoint [53].

Quinones

Mitomycin C is a widely used, quinone-based, anti-cancer therapeutic that functions via DNA cross-linking. In preclinical evaluation, it was noted that it had enhanced toxicity against hypoxic compared to normoxic cells, however the effect was minor [54]. This promoted development of other quinones with greater hypoxia selectivity, and of these, Porfiromycin (POR) and Apaziquone (EO9) represent the leading quinones as bioreductive prodrugs. Pre-clinical studies of POR in mouse EMT6 cells demonstrated superior hypoxic selectivity of POR over Mitomycin C, a result of lowered aerobic cytotoxicity [55]. POR showed additive toxicity to cells *in vitro* and more-than-additive (synergistic) cytotoxicity to solid murine tumors, in combination with irradiation [55]. However, although early clinical trials demonstrated POR had an acceptable toxicity profile in patients, a follow-up phase III trial concluded that POR was inferior to Mitomycin C as an adjunct to radiotherapeutic management of HNSCC [56].

EO9, demonstrates hypoxia selectivity via one-electron reduction however, in cells expressing the two electron oxidoreductase NQO1, two-electron reduction occurs. Therefore, normoxic off-target activation can take place, although this has led to efforts to utilize EO9 in tumors over-expressing NQO1 [57-59]. Early pre-clinical studies of EO9 demonstrated a unique anti-tumor profile when compared to Mitomycin C. *In vitro*, EO9 showed preferential cytotoxicity against solid tumors compared to leukemia cell lines, and to hypoxic versus aerobic cells [57]. EO9 also displayed higher hypoxic/normoxic differential cytotoxicity than Mitomycin C in solid mouse tumors, and both agents showed enhancement of response to radiation [60]. In a human glioblastoma mouse model, dosing of EO9 after radiation increased tumor doubling time by 8.5 days, more than twice the effect of EO9 or radiation treatment alone [61]. Furthermore, addition of EO9 to radiotherapy resulted in no significant increase in weight loss or normal tissue toxicity, leading the authors of the study to recommend that EO9 should be further explored as a radiosensitizer [61]. However, EO9 displays a poor

pharmokinetic profile, which has hindered evaluation in tumor types where local administration is not possible [58,62]. Therefore, quinones do not currently represent a class of clinically relevant hypoxia activated prodrugs. The rapid urinary clearance of EO9 has led to its evaluation for the treatment of bladder cancer in phase III trials, although results of these trials have not yet been published (NCT00598806, NCT01475266, NCT02563561).

Aromatic *N*-oxides

Tirapazamine (TPZ) is one of the best characterized HAPs. It is reduced by a number of one-electron oxidoreductases to form a TPZ radical that, in the absence of oxygen, progresses spontaneously to form benzotriazinyl and aryl radicals [63]. Two-electron reduction in the case of TPZ does not represent a mechanism of off-target activation, since it bypasses the formation of a TPZ radical to generate a metabolite with markedly lower toxicity than the active agent.

A number of pre-clinical and early phase clinical trials were conducted with TPZ in combination with various irradiation regimes and yielded promising results [64]. For example, combining TPZ with radiation has a synergistic effect on human cell lines (HRT, Na11 and MEWO) in a manner highly dependent on tumor oxygenation [65]. TPZ also showed enhancement of response of melanoma cell lines to irradiation, with minimal effect on the radio-sensitivity of normoxic cells [66-68]. In mouse models, pre-treatment with TPZ enhanced sensitivity of transplanted tumor cells to radiation [69,70]. A phase I clinical trial of TPZ with radiotherapy in the treatment of refractory solid tumors suggested that TPZ could safely be given concurrently and suggested it may be a radiosensitizer [71]. A later phase I clinical trial of TPZ with cisplatin and radiotherapy in SCLC concluded favorable survival of patients and acceptable toxicity of the drug [72]. A phase II study of TPZ with chemo-radiotherapy in locally advanced HNSCC reported 55% failure free survival in the treatment arm, a near-significant improvement in patient response [73]. In contrast, a concurrent phase II trial of TPZ with chemo-radiotherapy in HNSCC determined that TPZ increased hematological toxicity but did not improve outcomes in patients in the study [74]. This difference was perhaps because, despite attempts to stratify patient groups for levels of tumor oxygenation, there was an imbalance in the treatment arms

with more oxygenated tumors in the TPZ arm [74]. In addition, the dosing regimen differed between the studies, and the positive study was larger, giving greater power to detect an improvement.

Further phase III trials with TPZ in conjunction with cisplatin and radiation were also disappointing, concluding that administration of TPZ to patients with HNSCC led to no overall improvement in patient survival [75]. An important limitation which was highlighted following these failures is the excessive metabolic consumption of TPZ which limits its ability to reach poorly-perfused regions of tumor hypoxia. The phase III trial was also criticized for lack of stratification for patients with hypoxic tumors, and for the quality of radiation delivery [75,76]. The drug access issues with TPZ have been addressed by the development of SN30000, which has a more favorable diffusion profile and more efficient extravascular transport [77]. SN30000 is yet to enter clinical development.

Aliphatic *N*-oxides

AQ4N is the most clinically advanced aliphatic *N*-oxide. Of the bioreductive agents that have entered clinical trials, AQ4N is unique as it is reduced in an oxygen-sensitive two-electron reduction, mediating hypoxia selectivity without the redox cycling that is associated with reactive oxygen species generation [78]. The two-electron reduction of AQ4N is carried out by cytochrome P450 isozymes or nitric oxide synthase 2A, resulting in the formation of the topoisomerase inhibitor AQ4 [79,80]. The marked differences in physical properties between AQ4N and AQ4 prevent the former from stably interacting with DNA and the drug has been demonstrated to have significant activity in pre-clinical mouse models [81-84]. In tumor-bearing mice, AQ4N in combination with a single dose of radiation resulted in a marked increase in anti-tumor efficacy with no enhancement of toxicity to normal tissue compared to radiation alone [84]. AQ4N is active as a single agent in murine tumors, but in combination with radiation, AQ4N slowed tumor growth by over 40% compared to radiation alone [83]. This enhancement was affected with administration of the drug up to 16 hours before or after irradiation, suggesting that the active compound, AQ4 is stable in hypoxic cells and prevented their replication once cells in oxygenated regions were killed by radiation [83]. Positive results were also obtained in phase I clinical trials. No dose limiting effects were observed and no maximum tolerated dose was established in a study with patients with esophageal carcinoma treated with AQ4N

followed by radiotherapy [85]. Additionally, in a phase I clinical trial with patients with glioblastoma and head and neck tumors, AQ4N was selectively activated in hypoxic regions of solid tumors [86]. Unfortunately, development of this promising therapeutic has not progressed further than phase II trials. A phase II clinical trial of AQ4N with radiotherapy and temozolomide in glioblastoma began in 2006, but no results have been published (NCT00394628).

Limitations of DNA-targeted cytotoxins as bio-reductive prodrugs

The repeated failings of bioreductive prodrugs to fulfil their apparent pre-clinical potential calls into question which factors have limited their success in the clinic. Perhaps the most important limitation of bioreductive prodrugs in the context of these clinical trials is the failure to identify patients who are most likely to benefit from allocation to HAP therapy. The difficulties associated with identifying such patient subgroups are substantial, not least of which is the extensive variability in the incidence and severity of tumor hypoxia, even amongst relatively homogenous patient populations.

Tumor oxygenation can be directly measured using needle electrodes, and this technique was key to early work proving the association between hypoxia and treatment response [1-4]. However, this technique is limited to accessible tumors, and the availability of the equipment is limited to very few centers. An alternative approach is the infusion of exogenous tracers such as pimonidazole or radio tracers. Pimonidazole is reduced in the absence of oxygen and binds to macromolecules, this can be assessed in patient biopsies via immunohistochemistry [5,87]. Tumor hypoxia can be imaged via non-invasive methods such as magnetic resonance imaging and positron emission tomography (PET). A number of PET tracers are in use and in development, including [^{18}F]FMISO and [^{18}F]FAZA [6,7].

Hypoxia in tumors can also be evaluated by quantifying the changes in expression of specific hypoxia-responsive genes, for example CAIX, GLUT-1 and LOX [8-11]. However, an extensive review of endogenous markers of hypoxia suggested that no individual gene could be considered a definitive prognostic hypoxia marker and instead, the use of multiple gene expressions would give more accurate and specific hypoxia information [8]. A number of such cumulative gene responses,

termed hypoxia gene expression signatures, have been developed [12]. A hypoxia metagene based on the expression of 99 genes identified in a microarray study of HNSCC biopsies was shown to be an independent prognostic factor for recurrence-free survival [13]. The concept of hypoxia gene signatures is perhaps best exemplified by the 'Toustrup signature', a 15 gene hypoxia gene expression classifier with prognostic as well as predictive impact for the effect of hypoxia modifying therapy (nimorazole) in combination with radiotherapy [14].

In a study of HNSCC, assessment of 103 patients with T2-T4 larynx carcinomas was carried out with the hypoxia marker pimonidazole, which is reduced in the absence of oxygen, and subsequently imaged with immunohistochemistry [87]. Among tumors large variation in pimonidazole positivity and carbonic anhydrase IX (CAIX, a hypoxia-activated gene) were observed [87]. Even within this similar group of patients, the hypoxic tumor fraction varied twenty-fold. It follows that, for any meaningful patient stratification, future clinical trials must incorporate the imaging of tumor hypoxia to determine the presence, extent, and severity of hypoxia in each individual. Interestingly, the variability in tumor hypoxia observed in patients lies in contrast to that seen in xenograft models, in which tumor hypoxia is usually extensive. This over-representation of hypoxia in pre-clinical models may contribute to the exaggeration of bioreductive prodrug toxicity ratios in the lab.

The need to carry out such patient stratification in these clinical studies was demonstrated further by a sub-study of HNSCC patients randomly allocated to chemo-radiation therapy with or without TPZ in a phase II clinical trial [88]. Within the subgroup of patients, in whom substantial tumor hypoxia was found with fluoromisonidazole PET imaging, allocation to the TPZ-receiving group was associated with a significant reduction in loco-regional failure compared to allocation to a non-TPZ-containing regimen [88]. Despite these findings, even recent studies such as MAESTRO (NCT01746979) have not incorporated hypoxia imaging. It is likely that the absence of patient stratification in these trials is due to two considerations: the first is that many centers do not have the capacity to carry out screening for tumor hypoxia, and thus such a trial would be limited in center participation. Secondly, the expense involved with such stratification would likely be high. However, it seems apparent that some

form of patient stratification must be implemented for meaningful progress to be made in targeting tumor hypoxia.

An overwhelming challenge to HAPs is the difficulty in delivering these compounds to target cells. By definition, the target cells of HAPs are confined to hypoxic zones which are distant from functional blood vessels. Therefore, to reach hypoxic tumor regions, anticancer drugs must penetrate relatively long distances through the extravascular compartment, which is a particularly limiting for HAPs which require these conditions for activation. The importance of extravascular transport in tumors for the efficacy of HAPs was illustrated in a study of TPZ analogs in a multi-cellular layers (MCL) model, which showed substantial drug depletion in hypoxic regions due to diffusion limitations [89,90]. These studies revealed that for optimum prodrug efficacy, reduction kinetics need to be balanced to accommodate competing properties of metabolic stability (for tissue penetration) and metabolism to the cytotoxic metabolite (for cytotoxicity in hypoxic cells) [91,92].

In addition, regions of hypoxia are heterogeneous within tumors, therefore the 'bystander effect' is thought to be important for the activity of HAPs either for monotherapy or in combination with chemo- or radio-therapeutic agents to which moderately hypoxic cells are resistant [22]. In this scenario, the stability of the effector molecule following activation is an important consideration for it to be able to diffuse from the site of reduction and target nearby cells (which may not themselves be capable of prodrug activation) [21]. The bystander effect is thought to contribute to the anti-tumor activity of the HAP PR-104 in tumor xenograft models [22]. However, targeting cells with HAPs that are activated in severe hypoxia relies on these resistant population being adjacent to regions of anoxia, which may not be the case given the variation of perfusion and hypoxia in tumors [29]. The determination of such cases may be aided by emerging techniques for *in situ* functional imaging of intra-tumoral heterogeneity [93].

In addition to imaging hypoxia, an essential process in determining the suitability of a patient for therapy with bioreductive prodrugs is establishing that the oxidoreductive enzymes involved in prodrug metabolism are sufficiently expressed by the tumor. In this regard, some recent progress has been made; a recent study determined the one-electron reductases responsible for the activation of

TPZ and SN30000, and identified several such enzymes that could activate not only these bioreductive prodrugs, but also the hypoxia biomarker EF5 [77]. It follows that the use of EF5 as a biomarker might be able to inform clinicians of both the oxygenation state and reductase expression profile of a tumor with a single assay. Further work using genome-scale RNAi libraries in a reductase-focused screen identified P450 oxidoreductase as the principal determinant of cell sensitivity to SN30000, suggesting that expression of the enzyme itself should be explores as a predictive marker in clinical development of HAP [94].

Despite the appeal of this all-in-one solution, some issues remain with regard to the differences in pharmacokinetic properties between EF5 and TPZ/SN30000, highlighting the need for the parallel development of therapeutic agents and markers, which may be useful for indicating their utility in a given patient.

A further limitation of the clinical utility of bioreductive prodrugs lies in the design features that have guided their development. For traditional HAPs, activation in conditions of hypoxia is associated with the release of a potent DNA-damaging cytotoxin. Since this mechanism of cell killing resembles that used in traditional chemotherapeutic agents, these agents have limited use in combination therapy; importantly, toxicity overlap has frequently necessitated dose reductions during clinical trials [46]. The fact that overlapping toxicity with traditional chemotherapeutic agents represents a significant limitation to the utility of the above-described HAPs is increasingly being understood. This realization has ushered in a second generation of bioreductive prodrugs that, instead of releasing a potent DNA-damaging cytotoxin, are activated selectively in hypoxia to release a molecularly-targeted protein ligand (Figure 3). In this way, these prodrugs are capable of targeting promising cancer therapies to regions of tumor hypoxia, thereby allowing targeting of the most clinically-aggressive, treatment-refractory tumors.

Molecularly targeted bioreductive prodrugs

An early example of a molecularly targeted HAP was a prodrug of the poly(ADP-ribose) polymerase (PARP) inhibitor 5-bromoisoquinolinone [95]. The PARP1 protein is a nuclear protein that binds to sites of DNA damage and promotes repair [96]. Therefore, a hypoxia-activated PARP inhibitor could selectively sensitize hypoxic tumor cells to DNA damaging agents. Chemical reduction of this compound was shown, but no further testing for efficacy was reported. More recently, another hypoxia activated PARP inhibitor, an imide-N protected pyrrolocarbazole CEP-9722, was reported [97]. Pharmacokinetic studies revealed that the protected compound was converted to the active molecule in plasma in rats. The compound was further developed and is currently in phase II clinical trials for patients with advanced solid tumors or mantle cell lymphoma (NCT01345357, NCT00920595) [98,99].

In addition to DNA damaging agents, HAPs that enhance the efficacy of DNA alkylating agents in the hypoxic fraction of tumors have also been designed. O^6-alkylguanine-DNA alkyltransferase (AGT) is a DNA repair protein that removed alkyl groups from the O^6 position of guanine and therefore provides resistance to anticancer agents that alkylate this position [100]. An ethyl benzoate protected azeoaromatic prodrug of the ATG inhibitor O^6-benzylguanine was shown to be selectively reduced under hypoxic conditions and sensitize DU145 human prostate cancer cells to treatment with to the guanine O^6-alkylator laromustine [101].

An alternative approach, designing a HAP that could be used as a single agent therapeutic was recently demonstrated with the proof-of-concept development of a hypoxia inducible checkpoint kinase 1 (Chk1) and Aurora A kinase inhibitor (CH-01) [102]. Both of these kinases are important in cell-cycle progression and regulation, therefore, there are well-founded reservations toward inhibiting these targets systemically. Huge investment has been made into the development of Chk1 inhibitors but many clinical trials have been terminated due to cardiotoxicity [103,104]. By repurposing such compounds as bioreductive prodrugs, the therapeutic potential of these targets can be realized without the concurrent risks associated with their inhibition in normal, healthy tissues.

CH-01 achieves its hypoxia-selective activation through attachment of a 4-nitrobenzyl group to the hydroxyl terminus of the Chk1 inhibitor, thereby rendering it inactive until it is reduced under

hypoxic conditions [102]. Reduction of the compound to the active inhibitor and induction of a significant loss in viability was achieved in cancer cell lines exposed to hypoxia [102]. Inhibition of Chk1 leads to sensitization of human pancreatic adenocarcinoma cells to radiation through G_2 checkpoint abrogation and inhibition of homologous recombination repair [105]. Therefore, a hypoxia-activated Chk1 inhibitor could affect greater anti-tumor activity in combination with radiotherapy.

Another recent study has demonstrated success in combining a molecularly target bioreductive prodrug with radiotherapy. BCCA621C is a DNA-dependent protein kinase (DNA-PK) inhibitor, which has been attached to a nitroimidazole moiety to confer hypoxia selectivity. DNA-PK is highly important in facilitating non-homologous end joining [106], and hypoxic cells deficient in DNA-PK have been shown to be radio-sensitive compared to hypoxic DNA-PK proficient cells [107]. In preclinical testing, BCCA621C was found to be reduced to the active inhibitor selectively under conditions of severe hypoxia, in NCI-H460 human lung cancer cells and within these hypoxic cells it effected significant radiosensitization [107].

Currently, the most clinically advanced molecularly targeted HAP is TH-4000 (Tarloxotinib), a bioreductive pan-HER inhibitor. In normal cells, HER signaling pathways are involved in the regulation of cell growth and survival as well as adhesion, migration, differentiation, and other cellular responses [108]. Hyper-activation of HER family receptors is common in cancers and leads to downstream upregulation of MAPK, PI3K/AKT and JAK/STAT pathways which are linked to tumor progression, angiogenesis and metastasis [108]. Therefore, pan-HER inhibitors have been identified as promising therapeutics, but as with the aforementioned kinases, the importance of HER signaling in normal cellular function makes systematic inhibition unattractive. Under hypoxic conditions, TH-4000 undergoes one-electron reduction to a nitro radical anion that subsequently fragments to release an irreversible EGFR tyrosine kinase inhibitor (TKI) [109]. TKIs such as Erlotinib are already in use for NSCLC, in which mutations of EGFR are found in as many as a third of cases [110]. However, acquired treatment resistance and unfavorable adverse effect profiles are limiting to this treatment. TH-4000 attempts to address the limitations of conventional EGFR TKIs, and has shown significant

promise in preclinical trials [111,112]. Efficient metabolism of TH-4000 in hypoxia was demonstrated in a panel of human NSCLC cell lines and the HAP was shown to be more effective than Erlotinib in wild-type and EGFR-mutant NSCLC xenografts [111,112]. Two recent Phase II clinical trials of TH-4000 in NSCLC, squamous cell carcinoma of the head and neck (SCCHN) and squamous cell carcinoma of the skin (SCCS) included baseline HX4 PET imaging for hypoxia at select sites in the trial, representing steps towards more targeted trials for hypoxia activated drugs (NCT02454842 and NCT02449681). Unfortunately, patients with SCCHN or NSCLC did not achieve the primary interim response rate endpoint, and although the response observed in SCCS was encouraging, the trials were terminated by the funding company Threshold Pharmaceuticals [113].

Conclusions

Of the multiplicity of differences between malignant and normal tissues that have been described thus far, tumor hypoxia is perhaps the most striking that has yet to be exploited successfully in the clinic, despite long-standing efforts to do so. Despite this, positive clinical results with the combination of bio-reductive agents (TPZ, AQ4N and BCCA621C) and chemo-radiotherapy demonstrate the potential of this approach (Table 1). In assessing whether or not bioreductive prodrugs represent the future for targeting tumor hypoxia, it is important to note that there are a number of other methods by which tumor hypoxia can be targeted. Some of these methods, such as systemically targeting the metabolic or epigenetic changes associated with tumor hypoxia might hold significant potential [114,115].

The process of bioreductive prodrug development is complex, and has been recently described in detail [23]. It requires a near-exhaustive understanding of the parent drug, and further necessitates that the parent compound possesses an intrinsic amenability to the attachment of a bioreductive moiety. These requirements somewhat restrict the compounds which can be used to target regions of hypoxia in this way, and thus the development of systemically-acting agents that target tumor hypoxia will remain an important endeavor. However, where it is possible to target tumor hypoxia through HAPs, the advantages of non-systemic activity are evident.

Whilst bioreductive prodrugs are an elegant solution to targeting tumor hypoxia, they have thus far failed to translate their conceptual and pre-clinical attractiveness into clinical efficacy. The failures of bioreductive prodrugs can be attributed to three central limitations: first, there has been insufficient stratification of patients in clinical trials according to both the presence of tumor hypoxia and to the expression of the appropriate reductases. Second, the importance of optimizing HAPs for delivery to target cells in hypoxic regions, which are distant from functional vessels, has been largely ignored. Finally, the overlapping toxicities that exist between HAPs and traditional therapies limits the utility of these agents in combination therapy. The development of HAPs that are activated to release molecularly-targeted protein ligands rather than DNA-damaging cytotoxins represents an important step forward in overcoming these limitations, however, efforts to refine drug development in this field must be accompanied by attempts to optimize extravascular penetration and personalize the use of these agents clinically. In addition, the potential efficacy of these drugs in combination with radiotherapy to target the radioresistant regions of tumors is yet under-explored. If this can be achieved, personalized bioreductive prodrug therapy may well represent the future for targeting tumor hypoxia.

Figure Legends

Figure 1: Hypoxia-activated prodrugs could target radiation-resistant hypoxic cells in tumors. (**A**) Illustration of tumor hypoxia. As distance from the capillary increases, oxygen levels in tumor cells decrease and resistance to chemo and radio-therapeutics increases. (**B**) Illustration of potential benefits of combining HAPs with irradiation. Generally, cell killing by radiation is reduced as a function of distance from the capillary. In contrast, a hypoxia activated prodrug (HAP*)* should show the opposite activity profile. This leads to the prediction that a combination of standard treatment with HAPs should result in cell killing regardless of distance from the capillary. (**C**) The general mechanism of activation of hypoxia activated prodrugs by one and two electron (e^-) reductases. In the

presence of oxygen, the radical anion is quenched. In hypoxic conditions, further reduction results in activation of the prodrug. Two-electron reduction can lead to oxygen independent activation of the prodrug.

Figure 2: Structures of key DNA-damaging bioreductive prodrugs reviewed in this article. For brevity, transition metal complexes have been excluded, see Graf et. al (2012) and Renfrew (2014) for reviews of bioreductive transition-metal complexes as prodrugs [116,117].

Figure 3: Structures of the molecularly targeted hypoxia activated prodrugs discussed in this review. The bioreductive moiety is shown in grey.

Figure 4: Scheme of the reductive activation pathway of PR-104 and TH-302. (**A**) PR-104 is hydrolysed by systematic phosphatases to PR-104A that undergoes reduction to cytotoxic metabolites PR-104H and PR-104M. (**B**) TH-302 undergoes one-electron reduction to a radical anion which then fragments to give the active species Br-IMP.

Table 1: Summary of clinical studies of HAPs. $1e^-$: one-electron reduction. $2e^-$: two-electron reduction. HNSCC: Squamous cell carcinoma of the head and neck. SCLC: Small cell lung cancer. NSCLC: non-small cell lung cancer.

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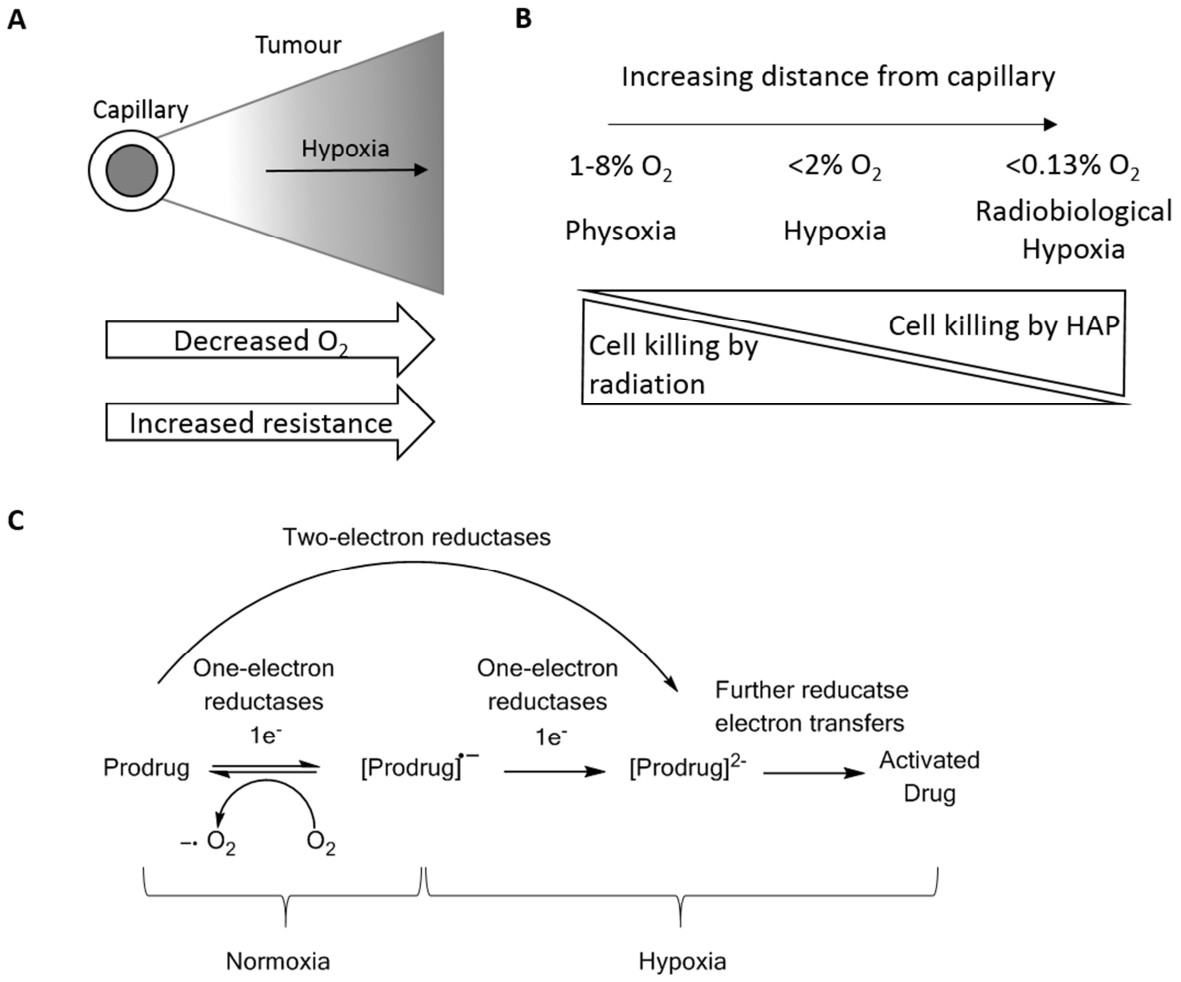
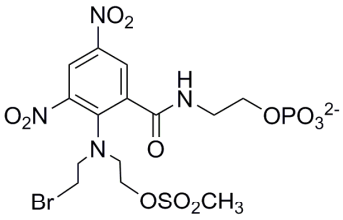
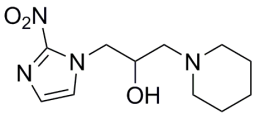
	Compound	Reduction mechanism	Study type	Target	Radiation	Outcome	Ref	Trial ref	Start/ *Publication date*
Nitro compounds	Pimonidazole	$1e^-$	Phase II	Cervical carcinoma	Y	No significant benefit	[36]		*(1993)*
	Etanidazole	$1e^-$	Phase I	HNSCC	Y	No significant benefit	[38]		*(1997)*
		$1e^-$		SCLC	Y	No significant benefit	[37]		*(1998)*
	Nimorazole	$1e^-$	Phase II	HNSCC	Y	Improved management of tumors	[41,42]	DAHANCA	*(1998)*
	PR-104	$1e^-/2e^-$	Phase II	SCLC	N	Trial terminated	[46]	NCT00544674	(**2007,** *2012*)
		$1e^-/2e^-$	Phase II	NSCLC	N	Trial terminated		NCT00862134	(**2009,** *2012*)
	TH-302 (Evofosafamide)	$1e^-$	Phase III	Pancreatic cancer	N	No significant benefit	[50]	NCT01746979	(**2012,** *2016*)
		$1e^-$	Phase III	Soft tissue carcinoma	N	No significant benefit	[50]	NCT01440088	(**2011,** *2016*)
		$1e^-$		Esophageal carcinoma	Y	Withdrawn prior to enrolment	[53]	NCT02598687	(**2015,** *2016*)
Quinones	Porfiromycin	$1e^-$	Phase III	HNSCC	Y	No significant benefit	[56]	NCT00002507	(**1999,** *2005*)
	Apaziquone (EO9)	$1e^-/2e^-$	Phase III	Bladder cancer	N	No published results		NCT00598806 NCT01475266 NCT02563561	(**2008**) (**2011**) (**2015**)
Aromatic *N*-oxides	Tirapazamine	$1e^-/2e^-$	Phase II	HNSCC	Y	Improvement in patient response	[73]	NCT00094081	(**2004,** *2005*)
		$1e^-/2e^-$	Phase II	HNSCC	Y	No significant benefit	[74]	NCT00002774	(**2000,** *2005*)
		$1e^-/2e^-$	Phase III	HNSCC	Y	No significant benefit	[75]	NCT00174837	(**2005,** *2010*)
Aliphatic *N*-oxides	AQ4N	$2e^-$	Phase I	Esophageal carcinoma	Y	No dose limiting effects	[85]		(*2008*)
		$2e^-$	Phase II	Gliobastoma	Y	No published results		NCT00394628	(**2006**)
Molecularly targeted	TH-4000 (Tarloxotinib)	$1e^-$	Phase II	NSCLC	N	No published results		NCT02454842	(**2015**)
		$1e^-$	Phase II	HNSCC	N	No published results		NCT02449681	(**2015**)


A
Tumour
Capillary
Hypoxia
Decreased O_2
Increased resistance
B
Increasing distance from capillary
1-8% O_2
<2% O_2
<0.13% O_2
Physoxia
Hypoxia
Radiobiological Hypoxia
Cell killing by HAP
Cell killing by radiation
C
Two-electron reductases
One-electron reductases
$1e^-$
One-electron reductases
$1e^-$
Further reducatse electron transfers
Prodrug
$[Prodrug]^{\bullet -}$
$[Prodrug]^{2-}$
Activated Drug
$O_2^{\bullet -}$
O_2
Normoxia
Hypoxia



PR-104A



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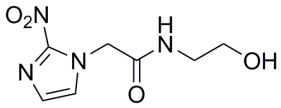
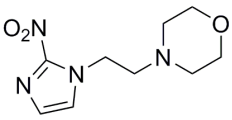
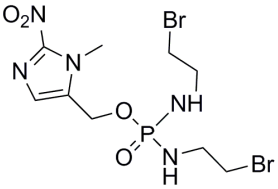


Etanidazole



Nimorazole



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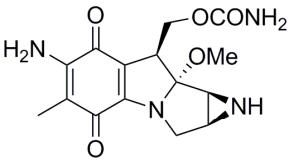
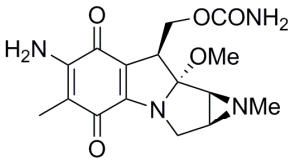


Mitomycin C



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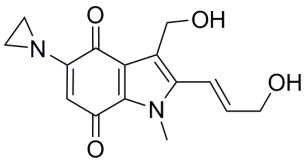

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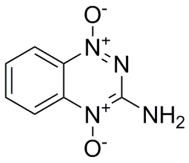

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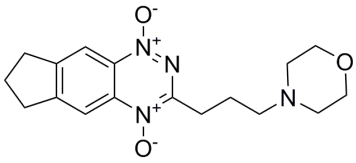

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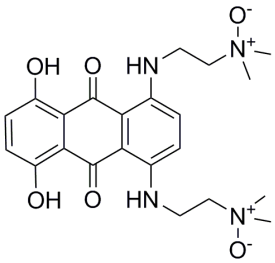

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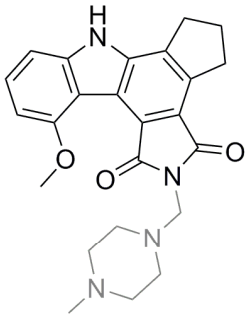

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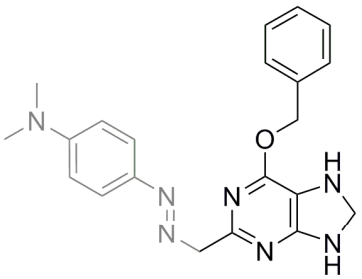
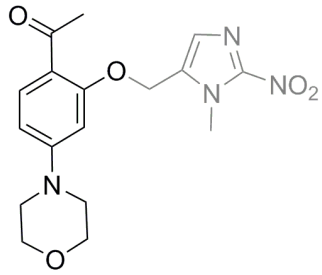


AGT inhibitor



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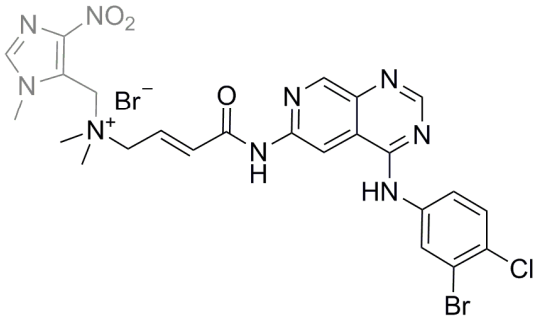

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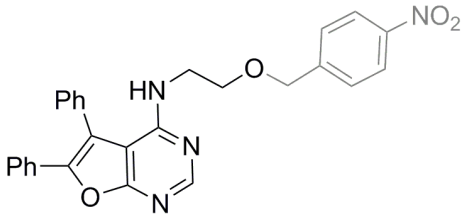

CH-01

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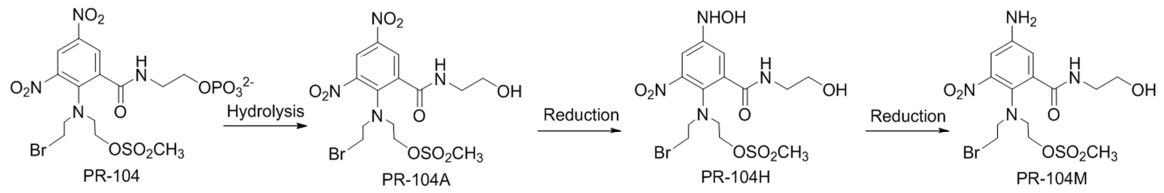

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