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Rational design of an AKR1C3-resistant analog of PR-104 for enzyme-prodrug therapy

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ABSTRACT

The clinical stage anti-cancer agent PR-104 has potential utility as a cytotoxic prodrug for exogenous bacterial nitroreductases expressed from replicating vector platforms. However substrate selectivity is compromised due to metabolism by the human one- and two-electron oxidoreductases cytochrome P450 oxidoreductase (POR) and aldo-keto reductase 1C3 (AKR1C3). Using rational drug design we developed a novel mono-nitro analog of PR-104A that is essentially free of this off-target activity *in vitro* and *in vivo*. Unlike PR-104A, there was no biologically relevant cytotoxicity in cells engineered to express AKR1C3 or POR, under aerobic or anoxic conditions, respectively. We screened this inert prodrug analog, SN34507, against a type I bacterial nitroreductase library and identified *E. coli* NfsA as an efficient bioactivator using a DNA damage response assay and recombinant enzyme kinetics. Expression of *E. coli* NfsA in human colorectal cancer cells led to selective cytotoxicity to SN34507 that was associated with cell cycle arrest and generated a robust 'bystander effect' at tissue-like cell densities when only 3% of cells were NfsA positive. Anti-tumor activity of SN35539, the phosphate pre-prodrug of SN34507, was established in 'mixed' tumors harboring a minority of NfsA-positive cells and demonstrated marked tumor control following heterogeneous suicide gene expression. These experiments demonstrate that off-target metabolism of PR-104 can be avoided and identify the suicide gene/prodrug partnership of *E. coli* NfsA/SN35539 as a promising combination for development in armed vectors.

1. Introduction

Gene-directed enzyme prodrug therapy (GDEPT) is an approach whereby cancer tropic vectors such as replication-competent viruses or bacteria are employed to deliver therapeutic genes to the tumor microenvironment. The exogenous gene typically introduces a new catalytic function into the tumor microenvironment that can confer conditional sensitivity to otherwise inert prodrugs [1]. The most widely studied nitroaromatic enzyme/prodrug combination for GDEPT is the nitroreductase (NTR) from *Escherichia coli*, NfsB, in combination with the prodrug CB1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide); see review [2] and references therein. Although efficacy was demonstrated with this combination in cell culture models [3,4] and tumor xenografts using replication-defective viruses [5,6], to date this combination has demonstrated limited utility in human clinical trials. Poor aqueous solubility of CB1954 [7], modest kinetics of CB1954 reduction by NfsB [8], and dose-limiting hepatotoxicity in humans [9] are thought to be possible reasons for the lack of efficacy observed.

The prodrug PR-104 (Fig. 1A), (2-((2-bromoethyl)(2-((2-hydroxyethyl)carbamoyl)-4,6-dinitrophenyl)amino) ethyl methanesulfonate phosphate ester) represents an alternative nitroaromatic substrate for NTRs. PR-104 is a water-soluble phosphate ester 'pre-prodrug' which undergoes facile conversion to the corresponding lipophilic alcohol PR-104A in plasma, and was initially designed and optimized as a hypoxia-activated prodrug (HAP) with

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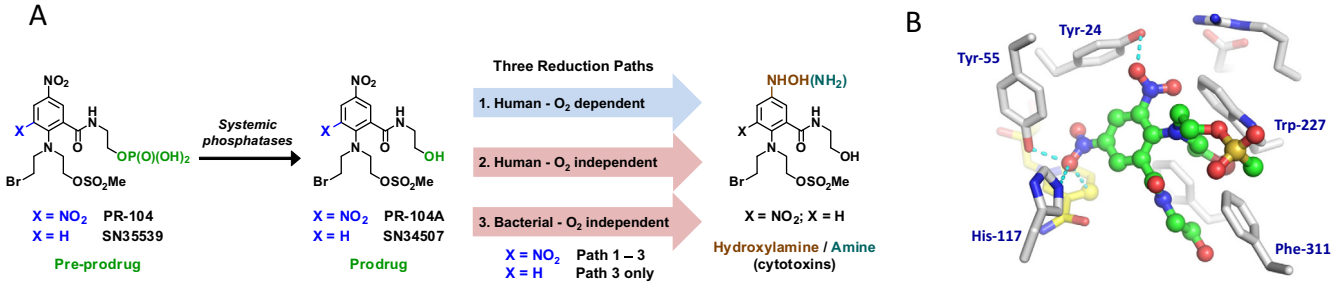

Fig. 1. Rationale for design of the prodrug SN34507: (A) Schematic of pre-prodrug PR-104 or SN35539 conversion to prodrug PR-104A and SN34507, respectively, with subsequent reduction to cytotoxic hydroxylamine or amine metabolites by up to three independent metabolic pathways; (B) Binding model of PR-104A in the active site of human aldo-keto reductase 1C3 indicating potential for hydrogen-bond formation between the *ortho* nitro group and tyrosine-24 residue (Figure generated with PyMol (Schrodinger)).

a bystander effect (diffusion of active metabolites from the cell of origin [10]). One-electron reduction of the *para* nitro group on PR-104A (relative to the nitrogen mustard) by human one-electron reductases such as cytochrome P450 oxidoreductase (POR) and methionine synthase reductase (MTRR) [11,12] creates a nitro radical anion intermediate. The radical anion is back-oxidized in the presence of molecular oxygen or, in the absence of oxygen (anoxia), will undergo further electron addition to form the DNA alkylating hydroxylamine (PR-104H) or amine (PR-104M). PR-104H and PR-104M can cause inter-strand DNA crosslinks with disruption of the replication fork upon mitosis, the most likely mechanism of toxicity [13,14]. Hypoxia selectivity was demonstrated in a range of cancer cell lines, with hypoxia cytotoxicity ratios (HCR; aerobic IC_{50} value divided by anoxic IC_{50} value) ranging from 5 to 100-fold [10]. However despite clear evidence for hypoxia-selective activation, atypical aerobic sensitivity of some cell lines to PR-104A suggested the presence of an aerobic reductase. The human oxidoreductase AKR1C3 (aldo-keto reductase member 1C3; P42330) was subsequently shown to reduce PR-104A in an oxygen independent (two-electron) manner by bypassing the formation of the oxygen-sensitive nitro radical intermediate, and is now accepted as the major determinant of aerobic PR-104A sensitivity [15].

AKR1C3 is a type 2 3α-HSD (hydroxysteroid dehydrogenase) and type 5 17β-HSD/prostaglandin F synthase [16], and is expressed at high levels in CD34+ myeloid progenitor cells [17]. Expression of AKR1C3 in these cells is a likely explanation for the underlying mechanism of the myelosuppression observed in humans after treatment with high doses of PR-104. Initial Phase I studies of PR-104 in patients with solid tumors determined that the primary dose-limiting toxicity was myelosuppression, predominantly neutropenia and thrombocytopenia, with delayed onset that was sometimes prolonged or irreversible [18,19]. This toxicity restricted the plasma exposure of PR-104A in humans to approximately one-third of the levels achievable in mice; mean plasma AUC of 39 μM-h is recorded in humans at 1100 mg/m^2 [18,19], compared to 120 μM-h in mice at 2144 mg/m^2 [20]. The inability of PR-104 to achieve sufficiently high plasma exposures in humans has limited its clinical utility in solid tumors.

Nevertheless the improved potency and bystander effect of PR-104A relative to CB1954 make it a potential candidate for GDEPT. PR-104A demonstrated a 9-fold improvement in anti-proliferative activity when compared to CB1954 in NfsB-expressing cells [21], and there is strong evidence for larger bystander effects from the dinitrobenzamide mustard class of compounds at tissue-like cell densities [22,23]. This evidence, taken together with the large therapeutic ratio demonstrated for PR-104A *in vitro* (WT:NfsB IC_{50} ratio of 2300-fold [21]), suggests that PR-104A remains a viable candidate for nitroreductase-based GDEPT. Consistent with this rationale, PR-104 has been evaluated in pre-clinical models with considerable success [24].

Ideally a GDEPT prodrug should be an exclusive substrate for a vector-encoded enzyme. PR-104A is subject to aerobic metabolism by human AKR1C3 and hypoxic metabolism by various one-electron oxidoreductases, which may limit the therapeutic specificity of the prodrug for NTR-armed vectors. In this study we have used rational drug design to develop more selective GDEPT prodrugs. We hypothesized that elimination of the *ortho* nitro of PR-104 would reduce the ability of AKR1C3 to recognize the dinitro motif. We also predicted that, in the absence of the electron-withdrawing effect of the *ortho* nitro group, the one-electron affinity ($E(1)$) of the remaining (*para*) nitro group would be lowered below that required for metabolism by human oxidoreductase enzymes [25], while retaining capacity for two-electron reduction by bacterial NTR enzymes [26]. The use of a single nitro group as the "bioreductive switch" should also provide more reactive/cytotoxic hydroxylamine and amine metabolites than PR-104H and PR-104M, as the residual deactivating influence (electron withdrawing nature) of the *ortho* nitro group would be absent. Based on the above hypotheses, we reasoned that replacement of the *ortho* nitro group of PR-104A with hydrogen would provide a suitable prodrug for selective activation by bacterial NTR enzymes. Here we report the novel PR-104A analog SN34507, and its water-soluble 'pre-prodrug' SN35539. We also identify the major *E. coli* NTR NfsA as an effective activating enzyme of SN34507 and demonstrate significantly improved therapeutic activity of this enzyme/prodrug combination over that of NfsA/PR-104 in an HCT116 colorectal cancer xenograft model.

2. Methods and materials

2.1. Chemicals

PR-104A was synthesized, purified and stored as previously reported [27,28]. PR-104 was supplied by Proacta, Inc. SN34037 was synthesized as previously reported [29].

2.2. Generation of a PR-104A binding model for AKR1C3

A three dimensional model of PR-104A was generated using the SKETCHER module followed by CONCORD(v.6.1.3) implemented within the SYBYL(v8.0.3) molecular modeling package (TRIPOS, St Louis, MO). A single low energy conformer was then generated by OMEGA(v2.2.1; OpenEye Scientific Software, Sante Fe, NM) using the MMFF94s forcefield, with the dielectric set at 80, the maximum number of conformers generated set to 30,000 and RMS set at 1. The low energy PR-104A conformer was then docked into the active site of the AKR1C3 enzyme with flufenamic acid

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