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Drug-DNA adducts as biomarkers for metabolic activation of the nitro-aromatic nitrogen mustard prodrug PR-104A

Alessia Stornetta[a,b], Kai-Cheng Kieren Deng[c], Sara Danielli[a], H.D. Sarath Liyanage[c], Shana J. Sturla[a,*], William R. Wilson[c,d,*], Yongchuan Gu[c,d]

[a] Department of Health Sciences and Technology, ETH Zurich, Schmelzbergstrasse 9, 8092 Zurich, Switzerland
[b] Masonic Cancer Center, University of Minnesota, 2231 6th Street SE, Minneapolis, MN 5545, United States
[c] Auckland Cancer Society Research Centre, The University of Auckland, Auckland, New Zealand
[d] Maurice Wilkins Centre for Molecular Biodiscovery, University of Auckland, Auckland, New Zealand

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ABSTRACT

PR-104A is a clinical-stage nitrogen mustard prodrug that is activated for DNA alkylation by reduction of a nitro group to the corresponding hydroxylamine (PR-104H) or amine (PR-104M). Metabolic reduction is catalysed by flavoreductases such as cytochrome P450 oxidoreductase (POR) under hypoxia, or by aldo-ketoreductase 1C3 (AKR1C3) independently of hypoxia. The unstable reduced metabolites are challenging to measure in biological samples, and biomarkers of the metabolic activation of PR-104A have not been used in the clinical evaluation of PR-104 to date. Here, we employ a selected reaction monitoring mass spectrometry assay for DNA crosslinks to assess the capacity of human cancer cells to bioactivate PR-104A. We also test whether the more abundant DNA monoadducts could be used for the same purpose. DNA monoadducts and crosslinks from PR-104A itself, and from its reduced metabolites, accumulated over 4 h in AKR1C3-expressing TF1 erythroleukaemia cells under hypoxia, whereas intracellular concentrations of unstable PR-104H and PR-104M reached steady state within 1 h. We then varied rates of PR-104A reduction by manipulating hypoxia or reductase expression in a panel of cell lines, in which AKR1C3 and POR were quantified by targeted proteomics. Hypoxia or reductase over-expression induced large increases in PR-104A sensitivity (inhibition of proliferation), DNA damage response (γH2AX formation), steady-state concentrations of PR-104H/M and formation of reduced drug-DNA adducts but not DNA adducts retaining the dinitro groups of PR-104A. The fold-change in the sum of PR-104H and PR-104M correlated with the fold-change in reduced crosslinks or monoadducts (R^2 = 0.87 for both), demonstrating their potential for assessing the capacity of cancer cells to bioactivate PR-104A.

1. Introduction

Despite important advances in targeting specific molecular changes in cancer cells, cytotoxic chemotherapy remains the mainstay in the management of most cancer types. Agents that induce highly cytotoxic DNA crosslinks, such as platinum complexes and nitrogen mustards, continue to be extremely important for cancer therapy [1]. However, the development and application of precision medicine tools for personalised use of these agents has lagged behind that of targeted therapy. In part, this shortcoming reflects that cell sensitivity to crosslinking agents depends on multiple determinants including pharmacokinetics, activity of plasma membrane transporters, metabolic activation/detoxification pathways and DNA repair phenotypes [2–4], whereas for molecularly targeted agents the presence of a specific oncogenic driver may predict sensitivity. Therefore, advancing precision-based therapeutic strategies for DNA-damaging agents requires delineating the quantitative relationships between exposure to the reactive cytotoxins, levels of DNA damage, and the DNA damage responses that determine cell fates.

PR-104A is a nitro-aromatic nitrogen mustard prodrug that is rapidly released from its water-soluble phosphate ester PR-104, which was the first hypoxia-activated prodrug of a nitrogen mustard to enter clinical trials [5–7]. PR-104A induces toxicity to cancer cells by generating DNA crosslinks upon reduction of the electron-withdrawing nitro group *para* to the mustard moiety, giving rise to the electron-donating hydroxylamine or amine group which activates the mustard in the corresponding metabolites PR-104H and PR-104M (Fig. 1). Evidence for the resulting formation of DNA interstrand crosslinks (ICLs) has been gained by use of the comet assay [8,9], and inferred from the hypersensitivity to PR-104A of cell lines with specific defects in ICL

* Corresponding authors at: Auckland Cancer Society Research Centre, The University of Auckland, Auckland, New Zealand (W.R. Wilson).
E-mail addresses: sturlas@ethz.ch (S.J. Sturla), wr.wilson@auckland.ac.nz (W.R. Wilson).

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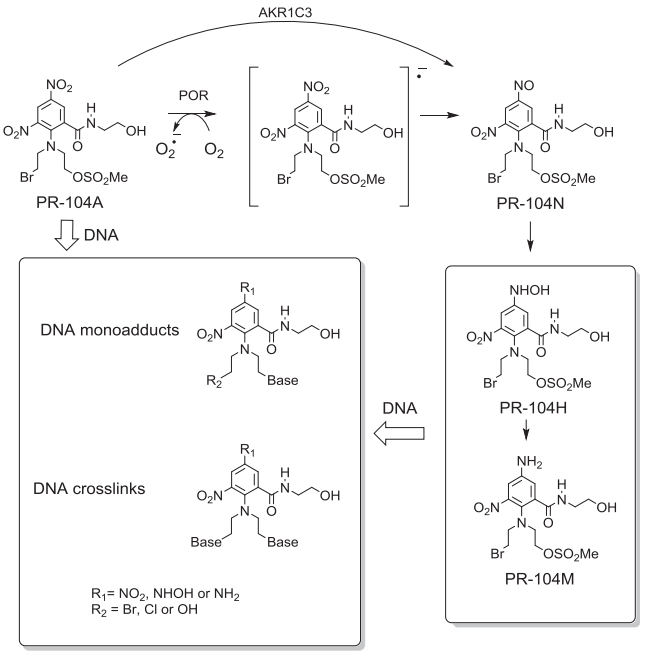

Fig. 1. Mechanism of metabolic activation of PR-104A by two-electron reduction catalyzed by AKR1C3 or one-electron reduction catalyzed by POR under hypoxia. DNA adducts are generated by PR-104A and by the more reactive reduced metabolites PR-104H and PR-104M.

repair [2,10,11].

The requirement for bioreductive activation provides a basis for tumour selectivity of PR-104A. The original design concept was to exploit tumour hypoxia through reduction by cytochrome P450 oxidoreductase (POR) and related one-electron reductases [12–14]. These flavoreductases generate a nitro radical that is rapidly re-oxidised by O_2 in normal tissue, but is reduced further to PR-104H and PR-104M in severely hypoxic cells (Fig. 1). In addition, PR-104A can be activated independently of hypoxia, but this requires the activity of aldo-ketoreductase 1C3 (AKR1C3), which catalyses the concerted two-electron reduction to the nitroso intermediate PR-104N (Fig. 1) [15–17]. The observation that hypoxia and AKR1C3 overexpression sensitise cells to the prodrug has led to preclinical and clinical studies to evaluate the activity of PR-104 in hepatocellular carcinoma [18,19] and in hypercellular bone marrow in acute leukaemias [20,21] due to the occurrence of both hypoxia and AKR1C3 expression in these cancer types. Additionally, the recent observation of high AKR1C3 expression in T-cell precursor acute lymphoblastic leukaemia (T-ALL) [17,22] and marked activity of PR-104 in T-ALL patient-derived xenografts [22] has stimulated interest in PR-104 for T-ALL therapy. However biomarker-guided clinical studies have not yet been undertaken for PR-104.

The identification of biomarkers diagnostic of capacity for metabolic activation of PR-104A is important for guiding clinical development of PR-104. The active metabolites PR-104H and M, for which we have developed sensitive LC–MS/MS assays [23], are direct indicators of metabolic activation but the instability of these compounds is a major limitation for their use as biomarkers. The resulting DNA adducts present a potential solution to the stability problem, and indeed there are significant data supporting the use of DNA adduct levels as biomarkers, with the most advanced clinical example being the use of microdosing and adduct analysis for oxaliplatin therapy [24].

PR-104A gives rise to many DNA adducts. Using a combined targeted and untargeted high-resolution/accurate-mass MS^3 adductomic approach, we comprehensively surveyed the DNA alkylation chemistry of PR-104A [25]. These data, derived from cell-free exposure of calf thymus DNA to PR-104A in the presence of AKR1C3/NADPH, indicated that PR-104A gives rise to mono and crosslinked DNA adducts directly, as well as from the metabolites PR-104H and PR-104M. When DNA isolated from PR-104A-exposed colon cancer cells (HT-29 cell line) was evaluated, 12 PR-104A-DNA monoadducts, three with reduced nitro groups, and a single PR-104A G-G nucleoside crosslink were observed. The relative abundances of these adducts could be determined using a selected reaction monitoring (SRM) mass spectrometric method with a triple quadrupole platform, using an internal standard mixture obtained by reacting calf thymus DNA with d_4-PR-104A in the presence of AKR1C3/NADPH and enzymatically hydrolysing the sample [26]. In this manner, changes in 25 mono- and 8 crosslinked adducts in DNA isolated from PR-104A-treated cells could be characterised as a function of AKR1C3 induction by pre-conditioning with the isothiocyanate sulforaphane, which we have shown to induce AKR1C3 in cancer cell lines but not in cell lines derived from healthy tissue [15,26].

Based on these preliminary observations, in the present study we tested whether relative quantitation of DNA crosslinks from PR-104A metabolites, using the above SRM method, provides a robust assay that reports the capacity of cancer cells to activate the prodrug. The approach was motivated in part by the expectation that the PR-104H/M DNA adducts will be more stable than the reactive metabolites and could be used as molecular dosimeters to integrate exposure to the active metabolites over time. A secondary objective was to assess whether the abundance of the more numerous monoadducts derived from PR-104H and PR-104M correlates with that of crosslinks, and whether measurement of reduced monoadducts might provide a more sensitive assay for PR-104A activation in individual tumours. To address these objectives we modulated rates of metabolic reduction of PR-104A by comparing cell lines with different levels of expression of AKR1C3 and POR (quantified by targeted proteomics), and by exposing them to PR-104A under both aerobic and anoxic conditions to modify activity of POR and other one-electron reductases. We assessed DNA adduct formation and intracellular concentrations of PR-104H and PR-104M, and also quantified the DNA damage response to crosslinks (formation of phospho-ser139 H2AX; γH2AX) and the anti-proliferative potency of PR-104A in each case. The new data demonstrate the use of DNA monoadducts and crosslinks from reduced PR-104A metabolites as biomarkers for the capacity of cancer cells to activate the prodrug.

2. Materials and methods

2.1. Reagents and chemicals.

PR-104A [27], its hydroxylamine metabolite PR-104H [8,27], amine metabolite PR-104M [2], and the AKR1C3 inhibitor SN34037 [28] were synthesised as previously described. Stock solutions were prepared in methanol (PR-104A), acetonitrile (PR-104H and PR-104M) or dimethyl sulfoxide (SN34037) and stored at −80 °C. Tetra-deuterated standards of the reduced metabolites (d_4-PR-104H and d_4-PR-104M) were prepared from d_4-PR-104A [29] in the same manner as the non-deuterated compounds. A DNA adduct internal standard reference mixture was prepared by incubating d_4-PR-104A with calf thymus DNA in the presence of AKR1C3 and NADPH as previously reported [26]. All other chemicals and enzymes were purchased from Sigma-Aldrich (St Louis, MO).

2.2. Cell lines and cell culture

All wild-type cell lines were from ATCC except for TF1, which was a gift from Dr. Julian Down, MIT. All lines were authenticated by short tandem repeat profiling. The stable transfectants HCT116/AKR1C3 [16] and HCT116/POR [14] have been reported previously. Cell lines were maintained in αMEM with 5% FCS, with addition of 1 μM puromycin for the transfectants. TF1 cells were grown in DMEM with 10% FCS and 5 ng/mL IL-3 (PeproTech, Rocky Hill NJ). All cell lines were cultured for < 3 months from frozen stocks confirmed to be Mycoplasma-free by PCR-ELISA (Roche Diagnostics, Basel, Switzerland).