Molecular determinants of T-cell acute lymphoblastic leukaemia sensitivity to the pre-prodrug PR-104

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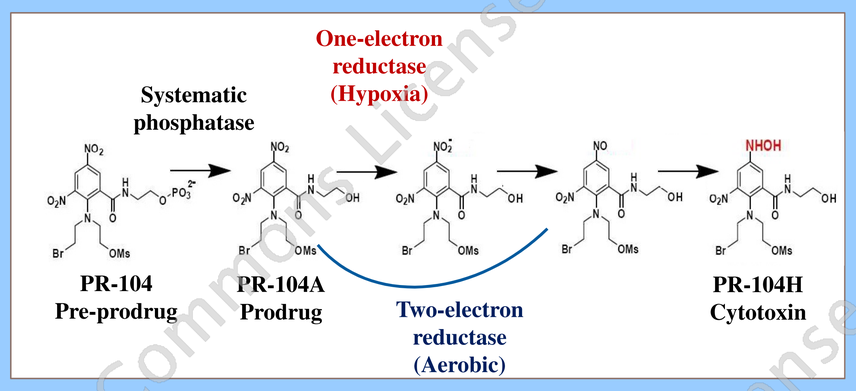
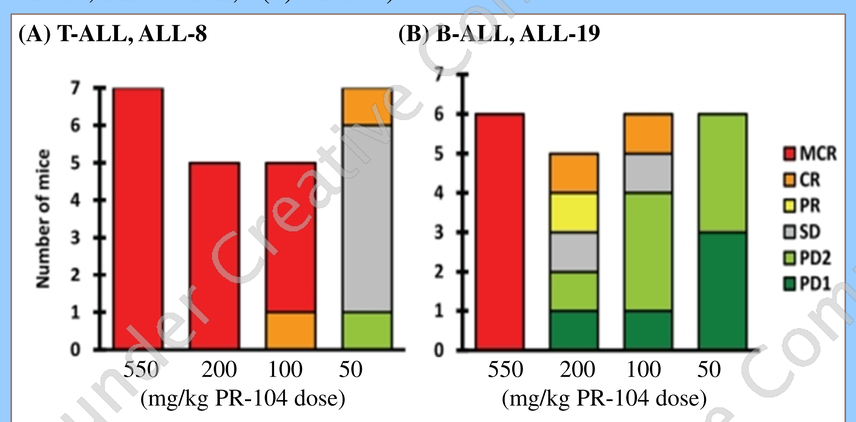
Introduction

Acute lymphoblastic leukemia (ALL) is the most common paediatric malignancy. While most children with ALL are cured, the prognosis for patients who relapse early is dismal and novel treatments are required. PR-104, currently in a phase I/II clinical trial for refractory or relapsed leukaemia, is a water soluble phosphate ester which is hydrolysed rapidly to its corresponding alcohol, PR-104A, *in vivo* . PR-104A is in turn activated by one-electron reductases under hypoxia to the DNA crosslinking mustard metabolites, PR-104H and PR-104M. PR-104A is also activated under aerobic conditions by the two-electron reductase aldo-keto reductase 1C3 (AKR1C3). The figure below illustrates the activation pathway of PR-104 (for additional details see http://www.fmhs.auckland.ac.nz/sms/acsrc/etg/pr104.aspx).



PR-104 was previously shown to exert remarkable in vivo efficacy when tested against a panel of T- and B-ALL xenografts in NOD/SCID mice by the Pediatric Preclinical Testing Program using a dose schedule of 550 mg/kg q7dx6 (Houghton et al., 2011, Pediatr Blood Cancer, 57: 443-453). Subsequent testing at attenuated doses (50-200 mg/kg) showed steep reduction in efficacy against a B-ALL (Panel B below), but not a T-ALL (Panel A), xenograft (Benito et al., 2011, PLoS ONE, 6(8):e23108).



Distribution of Objective Response Measures for individual mice engrafted with ALL-8 (A) and ALL-19 (B) at each dose of PR-104.

The purpose of this study was to elucidate underlying mechanisms for differences in responses of B- versus T-ALL to PR-104.

Methods

Immunoblot analysis. Whole cell lysates were prepared from freshly thawed xenograft cells. Proteins were separated by SDS-PAGE and transferred to PVDF membrane and probed with an anti-AKR1C3 mouse monoclonal antibody (Sigma). ECL detection of proteins was quantified using Quantity One-4.6.9, and AKR1C3 expression was normalised to actin and a HeLa cell control lysate.

mRNA expression analysis. Total RNA was extracted using an RNeasy Mini Kit (QIAGEN) and reverse transcribed using M-MLV reverse transcriptase (Invitrogen). Primer and probes for AKR1C3 were purchased from Applied Biosystems. AKR1C3 expression was quantified using real-time PCR (ABI Prism 7500) and normalized to a internal control, Elongation factor-1α (EF-1α).

In vitro culture and cytotoxicity assays. Xenograft cells were retrieved from cryostorage and resuspended in QBSF-60 medium (Quality Biological, Gaithersburg, MD) supplemented with Flt-3 ligand (20 ng/ml). For cytotoxicity experiments cells were equilibrated in medium overnight before addition of PR-104A at final concentrations of up to 100μM. After a 48-hr exposure, drug sensitivity was assessed using the alamar blue assay. Cell viability was expressed as a percentage of solvent-treated controls.

Enzymatic activity of AKR1C3. Xenograft cells were resuspended in X-VIVO™ 10 medium without phenol red (BioWhittaker) supplemented with Flt-3 ligand (20 ng/ml) and BIT 9500 Serum Substitute (STEMCELL Technologies), and equilibrated in medium for 3 h. The AKR1C3-specific inhibitor, SN34037, was then added to the required wells at a final concentration of 1 μM. Following a 1 h incubation, the fluorogenic probe coumberone was added at a final concentration of 10 μM. AKR1C3 activity was expressed as SN34037-sensitive coumberol formation over 24 h (μM/10^6 cells) in relation to a coumberol calibration curve.

Statistical methods. Differences between groups were assessed by one-way ANOVA, and the degree of correlation between groups by Pearson's correlation. The level of significance was set to 0.05.

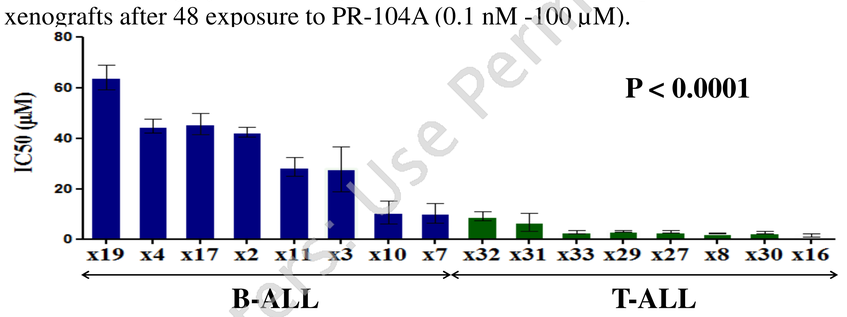
Acknowledgments

Supported by NOI-CM-91001-03 from the National Cancer Institute USA, and by Proacta. Children's Cancer Institute Australia is affiliated with the University of New South Wales and the Sydney Children's Hospitals Network.

Results

1. PR-104A is more effective against T-ALL than B-ALL xenografts *in vitro*

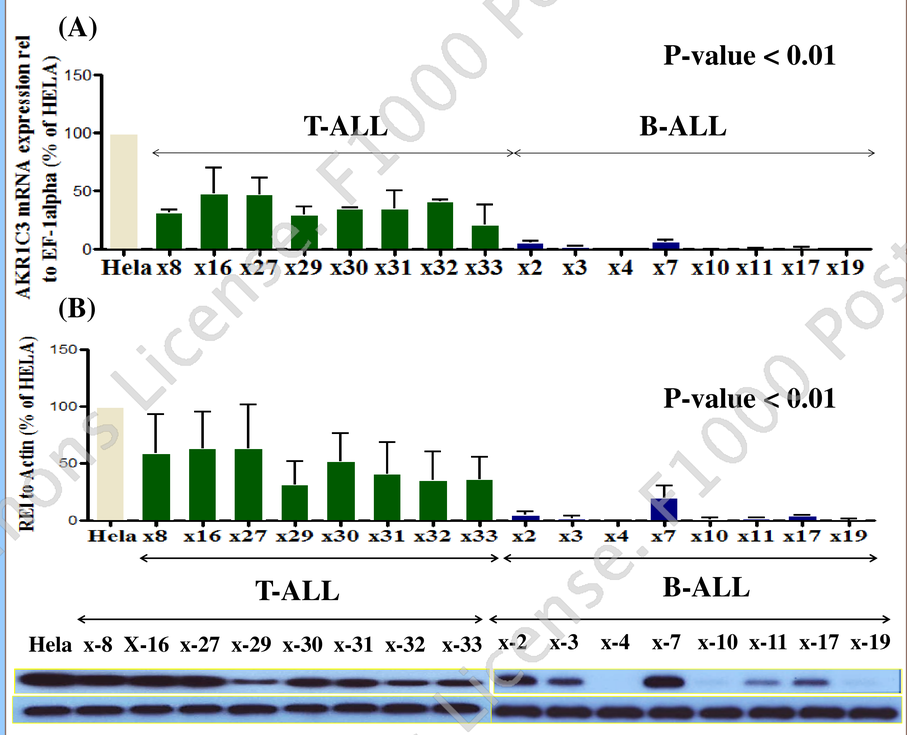
PR-104A was highly effective against T-ALL xenografts compared to B-ALL xenografts after 48 exposure to PR-104A (0.1 nM -100 μM).



IC50 values ranging from 1.54-9.02 μM for T-ALLs and 10.32-64.11 μM for B-ALLs. The median IC50 value of B-ALLs (34.35 μM) was 8.97-fold more than that of the T-ALLs (3.82 μM).

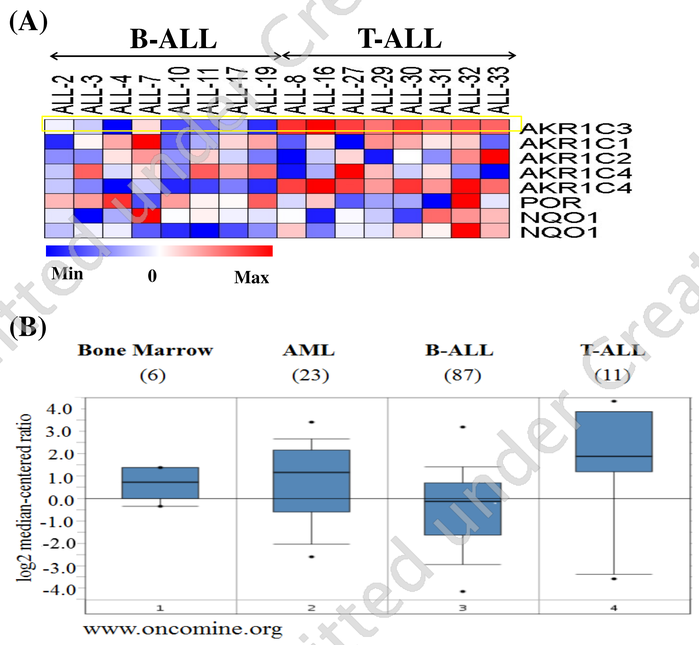
3. AKR1C3 is highly expressed in T-ALL compared to B-ALL xenografts at both the mRNA and protein level

To elucidate underlying mechanisms for differences in responses of B- versus T-ALL to PR-104, AKR1C3 protein and mRNA levels were determined for the panel of 8 B- and 8 T-ALL xenografts. AKR1C3 showed significantly higher expression in T- compared to B-ALLs at both the mRNA and protein level (P-value < 0.01) which was significantly correlated with PR-104A IC50 values (P-value = 0.0003), indicating that AKR1C3 is an important determinant of PR-104 sensitivity.



(A) mRNA expression levels of AKR1C3 in xenografts, normalised to EF-1 alpha; (B) protein expression levels of AKR1C3 in xenografts relative to Actin; (C) correlation between AKR1C3 mRNA and protein expression; (D) correlation between AKR1C3 protein and PR-104A sensitivity.

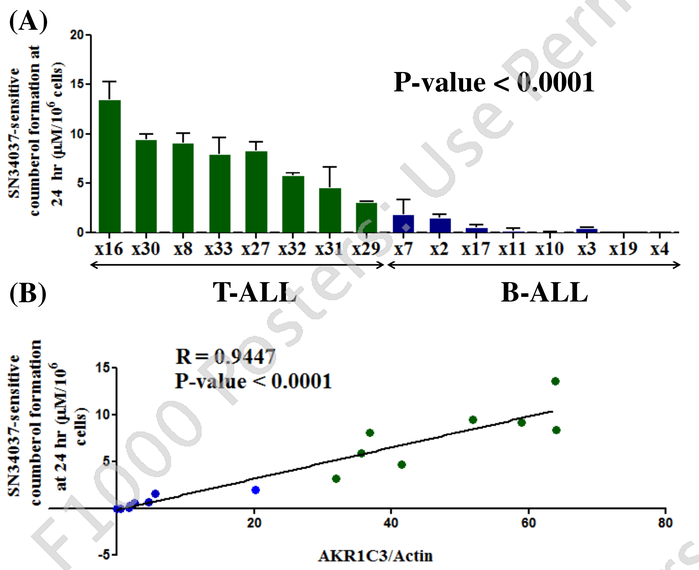
2. AKR1C3 mRNA is highly expressed in T-ALL compared to B-ALL xenografts and patient samples



(A) AKR1C3 and related gene expression in T- and B-ALL xenografts in heatmap format. (B) AKR1C3 expression in primary bone marrow samples (from www.oncomine.org).

4. AKR1C3 shows higher activity in T-ALLs, and activity correlates with protein expression

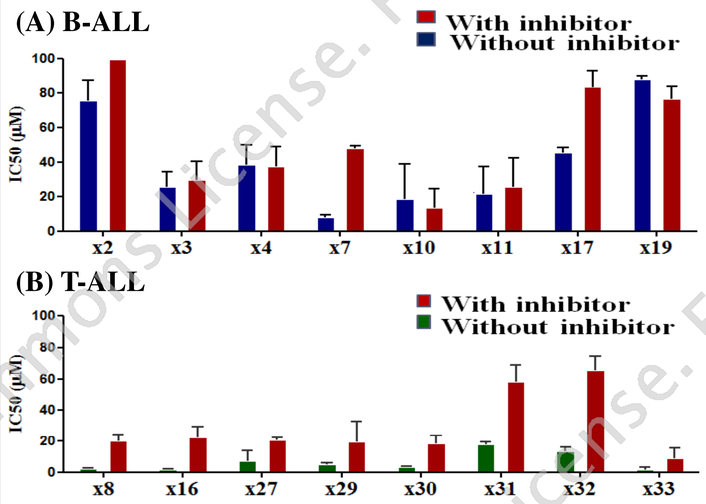
AKR1C3 activity was significantly higher in T-ALL compared with B-ALL xenografts, and protein expression significantly correlated with enzymatic activity as measured by an *in vitro* coumberone fluorescence assay (P<0.0001).



(A) AKR1C3 enzymatic activity; (B) Correlation between AKR1C3 protein expression and activity.

5. Specific AKR1C3 inhibitor markedly increases PR-104A IC50 in T-ALLs

Using an AKR1C3 specific inhibitor IC50 values increased by 6.3-fold across all 8 T-ALL xenografts, which indicates that AKR1C3 is a target for PR-104A and AKR1C3 detection may identify individual patients who may respond well to PR-104.



IC50 values for B-ALL (A) and T-ALL (B) xenografts after 48-hr exposure to PR-104A without and with AKR1C3 specific inhibitor SN34037 (1-hr incubation).

Conclusions

- **AKR1C3 is highly expressed in T-ALL xenografts and primary patient samples in contrast to B-ALL**
- **AKR1C3 expression correlates with PR-104A response *in vitro* in T-ALL and B-ALL xenografts**
- **AKR1C3 inhibition results in resistance to PR-104A in T-ALL xenografts**
- **Our findings indicate that AKR1C3 expression is an important determinant of *in vitro* and *in vivo* sensitivity to PR-104/PR-104A, and have implications for the clinical development of PR-104 since patients with T-ALL may represent a particularly responsive population**