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p21^{WAF1} modulates drug-induced apoptosis and cell cycle arrest in B-cell precursor acute lymphoblastic leukemia

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p21^{WAF1} is a well-characterized mediator of cell cycle arrest and may also modulate chemotherapy-induced cell death. The role of p21^{WAF1} in drug-induced cell cycle arrest and apoptosis of acute lymphoblastic leukemia (ALL) cells was investigated using p53-functional patient-derived xenografts (PDXs), in which p21^{WAF1} was epigenetically silenced in T-cell ALL (T-ALL), but not in B-cell precursor (BCP)-ALL PDXs. Upon exposure to diverse cytotoxic drugs, T-ALL PDX cells exhibited markedly increased caspase-3/7 activity and phosphatidylserine (PS) externalization on the plasma membrane compared with BCP-ALL cells. Despite dramatic differences in apoptotic characteristics between T-ALL and BCP-ALL PDXs, both ALL subtypes exhibited similar cell death kinetics and were equally sensitive to p53-inducing drugs *in vitro*, although T-ALL PDXs were significantly more sensitive to the histone deacetylase inhibitor vorinostat. Transient siRNA suppression of p21^{WAF1} in the BCP-ALL 697 cell line resulted in a moderate depletion of the cell fraction in G1 phase and marked increase in PS externalization following exposure to etoposide. Furthermore, stable lentiviral p21^{WAF1} silencing in the BCP-ALL Nalm-6 cell line accelerated PS externalization and cell death following exposure to etoposide and vorinostat, supporting previous findings. Finally, the Sp1 inhibitor, terameprocol, inhibited p21^{WAF1} expression in Nalm-6 cells exposed to vorinostat and also partially augmented vorinostat-induced cell death. Taken together, these findings demonstrate that p21^{WAF1} regulates the early stages of drug-induced apoptosis in ALL cells and significantly modulates their sensitivity to vorinostat.

Introduction

As a cyclin-dependent kinase (CDK) inhibitor, p21^{WAF1} can cause cell cycle arrest at both the G1-S and G2-M cell cycle phases.[1,2] p21^{WAF1} has been characterized as a tumor suppressor[3] primarily due to its role in mediating cell cycle arrest by both p53-dependent and p53-independent processes.[4] Under certain conditions p21^{WAF1} can also function as an anti-apoptotic factor. Up-regulation of p21^{WAF1} can efficiently protect various cell types from apoptosis,[5,6] whereas suppression of p21^{WAF1} results in increased sensitivity to apoptosis induced by a range of cytotoxic drugs and γ-irradiation.[7-9] Specifically, in malignant hematopoietic cells inhibition of p21^{WAF1} by various means, including antisense oligonucleotide transfection, increased sensitivity to cytotoxic drugs such as paclitaxel or cytarabine.[10,11] Conversely, overexpression of p21^{WAF1} in a range of leukemia and lymphoma cell lines conferred some resistance to cytarabine or etoposide.[10,12,13] However, in a recent study deletion of *cdkn1a* in a murine Eμ-Myc lymphoma model did not sensitize lymphoma cells to histone deacetylase inhibitor (HDAC)-induced apoptosis,[14] highlighting that questions remain over the proposed strength of the anti-apoptotic role of p21^{WAF1} in hematopoietic cells.

The interplay between cell cycle inhibition and apoptosis initiation is regulated in certain settings by the p53 protein, which ultimately determines the relative sensitivity of tumor cells to chemotherapy induced cell death.[15] One mechanism that has been recently demonstrated to control the switch between cell cycle arrest and death under DNA damaging conditions is the binding of DNA-dependent protein kinase, catalytic subunit (DNA-PKcs) to p53 and their recruitment to p53-responsive elements on the p21^{WAF1} promoter leading to rapid loss of p21^{WAF1} transcription and increased apoptosis.[16] *TP53* mutations represent another mechanism that leads to drug resistance and perturbed initiation of apoptosis in cancer cells,[17] although in diseases such as childhood acute lymphoblastic leukemia (ALL),[18,19] apoptosis can also be inhibited by alternative mechanisms, including ATM inactivation,[20] overexpression of Hdm-2,[21] expression of anti-apoptotic proteins,[22] or dysfunction in the p53-p21^{WAF1} axis.[23,24] Upregulation of p21^{WAF1} and disruption of the cytotoxic response can occur irrespective of *TP53* gene mutations.[20,24,25] In addition, the induction of p21^{WAF1} that

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occurs after exposure to various cytotoxic stimuli can inhibit the apoptosis process in malignant hematopoietic cells[26-28] and solid tumor cells.[5,29] In the clinical setting, elevated $p21^{WAF1}$ expression has been associated with chemotherapy resistance and poor prognosis in acute myeloid leukemia,[30,31] while an association with $p21^{WAF1}$ induction and poor clinical outcome in ALL has been proposed.[32]

Mechanisms proposed to explain the anti-apoptotic role of $p21^{WAF1}$ include transcriptional regulation of anti-apoptotic genes,[33] inhibition of CDKs that are involved in activation of caspases integral to apoptosis downstream of mitochondrial disruption,[9] or direct inhibition of pro-apoptotic proteins, such as procaspase-3, caspase-8 or apoptosis signal-regulating kinase 1.[3,33] Inhibition of CDKs has also been demonstrated to negatively affect caspase activation[9] and chromatin condensation.[34]

Cell death pathways induced by chemotherapy drugs include apoptotic and non-apoptotic processes. Apoptosis is influenced by caspase activity, establishing the necessary characteristics of the early stages of apoptosis such as phosphatidylserine (PS) externalization and condensed nuclei.[35] Though caspase-independent forms of cell death exist, the induction of apoptosis is thought to be the predominant pathway to cancer cell destruction. However, caspase activity is not always necessary for apoptosis, and other death pathways are initiated depending on cell type and cytotoxic stimuli.[36,37] For example the apoptosis executioner, caspase-3 may stimulate the repopulation of cancer cells by increasing inflammatory signals and activating pro-survival pathways in other malignant cells.[38,39]

With $p21^{WAF1}$ having an anti-apoptotic role in response to certain cytotoxic agents, inhibition of $p21^{WAF1}$ has been considered as a strategy for cancer treatment to sensitize cells toward apoptosis after chemotherapy exposure.[40] The Sp1 inhibitor, terameprocol, has been previously demonstrated to inhibit $p21^{WAF1}$ expression[41] and can be utilized to demonstrate any impact of $p21^{WAF1}$ inhibition on cell death.

This study examines the influence of $p21^{WAF1}$ on the cell death pathways of ALL cells after exposure to chemotherapeutic drugs. Various models of ALL were utilized, including patient-derived xenografts (PDXs) with epigenetically silenced $p21^{WAF1}$ in p53-functional T-ALL samples, transient siRNA and stable lentiviral knockdown of $p21^{WAF1}$ expression in BCP-ALL cell lines and pharmacological modulation of $p21^{WAF1}$ induction by terameprocol.[41] Our results show that $p21^{WAF1}$ exerts a significant influence on the kinetics of apoptosis mediated by chemotherapeutic drugs, but does not markedly influence in vitro sensitivity to p53-inducing agents or non-apoptotic characteristics of cell death.

Results

T-ALL cells exhibit increased apoptotic characteristics compared with BCP-ALL cells following exposure to cytotoxic drugs

While $p21^{WAF1}$ induction has been demonstrated to inhibit apoptosis in various cancer histotypes with a range of sensitivity to cytotoxic stimuli,[5-8,10-14,28,42] the anti-apoptotic role of $p21^{WAF1}$ in p53-functional, chemotherapy-sensitive hematopoietic cells has still not been fully elucidated. We assessed the *in vitro* apoptotic responses of T-ALL and BCP-ALL PDXs and cell lines following exposure to etoposide or vorinostat. The p53 status and $p21^{WAF1}$ responses of the PDXs used in this study have previously been determined and are summarized in Table 1.[23] To evaluate the apoptotic response of lymphoid leukemia cells to cytotoxic agents that induce $p21^{WAF1}$, we assessed caspase-3/7 activity and PS externalization in PDX cells and ALL cell lines exposed to 5 μM of etoposide or vorinostat, with or without prior incubation with the pan-caspase inhibitor z-VAD-FMK for up to 48 h of drug exposure. Caspase-3/7 activity of 2 T-ALL PDXs increased to 2-5 OD450 units/mg protein from <0.6 OD450 units/mg protein within 6 h of exposure to etoposide (**Fig. 1A-B**) or vorinostat (**Fig. S1A-B**), while caspase activity in the 2 BCP-ALL PDXs and 1 BCP-ALL cell line (Nalm-6; data not shown) remained equal to or below 2 OD450 units/mg protein after drug exposure (**Fig. 1C-D** and **Fig. S1C-D**). Caspase-3/7 activity remained high in the T-ALL PDXs for 48 h, while exhibiting much reduced activity in the BCP-ALL PDXs (**Fig. 1A-D** and **Fig. S1A-D**). z-VAD-FMK, effectively inhibited caspase-3/7 activity in all PDX lines tested (**Fig. 1A-D** and **Fig. S1A-D**).

Consistent with the difference in caspase-3/7 activity between T-ALL and BCP-ALL PDXs, PS externalization, as assessed by annexin V binding on PI^- cells, was also increased in T-ALL PDXs and cell lines (**Fig. 1E-F**, **Fig. S1E-F**, **Fig. S2A-D**). T-ALL PDXs and the Molt-4 cell line exhibited rapid (within 6-12 h) PS externalization in up to 60% of cells, whereas BCP-ALL PDXs and Nalm-6 cells showed <25% of PS externalization over the 48 h test period, and all with delayed kinetics compared with T-ALL cells. An exception was the Reh cell line, where ~35% of cells exhibited PS externalization following etoposide exposure (**Fig. 1F**), albeit with delayed kinetics. Despite dramatic differences in caspase-3/7 activation and PS externalization, both B- and T-cell lineages appeared equally sensitive to drug-induced cell death as evidenced by the proportion of PI^+ cells. Cell death kinetics in T-ALL PDXs was only marginally accelerated compared to BCP-ALL PDXs over the first 12 h of exposure to 5 μM of each drug, but was almost identical thereafter (**Fig. S3**).

We next compared the apoptotic responses between the 2 ALL lineages exposed to cytotoxic drugs that do not specifically induce $p21^{WAF1}$ expression. ALL-8 (T-ALL PDX) cells exhibited a prominent increase in caspase-3/7 activity and PS externalization compared with ALL-3 (BCP-ALL PDX) cells following exposure to the glucocorticoid dexamethasone and the kinase inhibitor staurosporine (**Fig. S4A-D**). However there was no substantial difference in the rate of loss of cell viability (**Fig. S4E-F**). Thus, T-ALL cells exhibit increased apoptotic characteristics compared with BCP-ALL cells when exposed to a broad range of cytotoxic drugs, despite demonstrating comparable kinetics of cell death.

To determine whether the differences in apoptotic characteristics exhibited by T-ALL and BCP-ALL cells were associated with

subtle variations in drug responses, we tested their sensitivity to a range of concentrations of etoposide, vorinostat and the Hdm-2 inhibitor nutlin-3 using the MTT cytotoxicity assay. While equally sensitive to etoposide and nutlin-3, the IC_{50} values of T-ALL PDXs treated with vorinostat were significantly lower than BCP-ALL PDXs ($p = 0.03$) (**Table 1**, **Fig. S5**), demonstrating increased sensitivity of T-ALL PDXs to the cell death inducing effects of vorinostat.

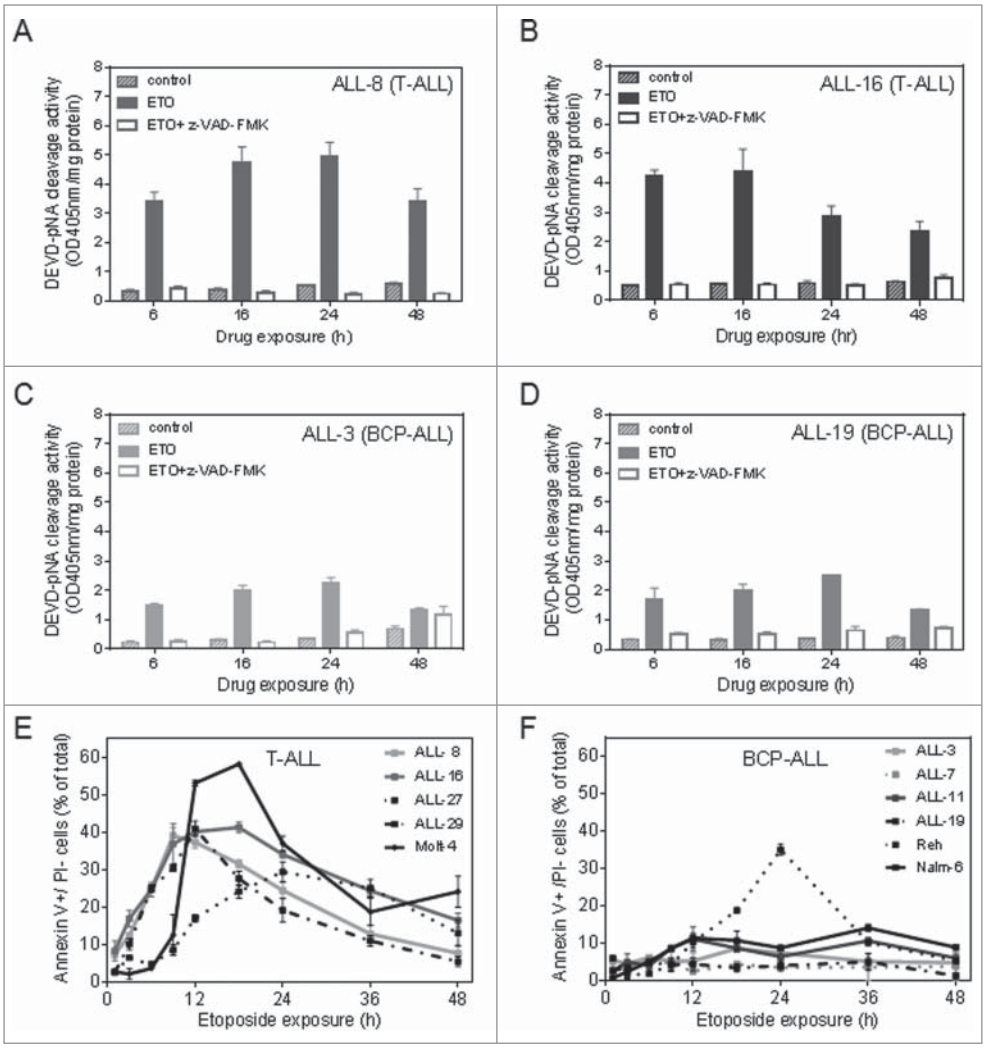

Figure 1. The effect of etoposide on caspase activity and PS externalization in T-ALL and BCP-ALL PDXs and cell lines. T-ALL PDX cells [A, ALL-8; B, ALL-16] and BCP-ALL PDX cells [C, ALL-3; D, ALL-19] were exposed to solvent control or etoposide (ETO) for 6 h, 16 hr, 24 h and 48 hr, with or without pre-treatment with z-VAD-FMK (75 μM, 16 h). Caspase-3/7 activity is expressed as DEVD-pNA cleavage activity. Each bar represents the mean caspase activity ± SEM of 3 independent assays. PS externalization on the outer membrane of cells was determined by staining cells with annexin V-FITC and PI. Collated data of annexin V+/PI- cells over 48 h of etoposide exposure are shown in E (T-ALL samples) and F (BCP-ALL samples). Each point represents the mean % of annexin V+/PI^- cells ± SEM as calculated from 3 biological replicates.

$p21^{WAF1}$ knockdown enhances drug-induced apoptosis in BCP-ALL cells

To determine whether the previously reported difference in $p21^{WAF1}$ expression and induction between T-ALL and BCP-ALL PDXs accounted for the divergence in apoptotic responses to cytotoxic drugs, we used knockdown approaches in BCP-ALL cell lines. First, we utilized siRNA to efficiently knockdown $p21^{WAF1}$ expression in the 697 cell line for a 48 h time period (**Fig. 2A**). $p21^{WAF1}$ knockdown resulted in a marked increase in early apoptotic (annexin V^+/PI^-) cells following etoposide treatment (**Fig. 2B**) although, consistent with the PDX data described above, the proportion of non-viable (PI^+) cells remained unaffected (**Fig. 2C**). $p21^{WAF1}$ knockdown also had little impact on etoposide-induced accumulation of cells in G2/M or depletion from S phase, although it did result in a modest depletion of cells from G1 and increase in the sub-G1 fraction (**Fig. 2D**).

We also tested the efficiency of microRNAs cloned into a lentiviral vector for stable $p21^{WAF1}$ knockdown in Nalm-6 cells (**Table S1**). Lentiviral transduction with miRNA #2 mediated the most effective knockdown of $p21^{WAF1}$ protein in Nalm-6 cells (**Fig. 3A**) and this construct was used in subsequent experiments. Mean intracellular $p21^{WAF1}$ protein levels assessed by flow cytometry were increased by 5.5–6.0 fold following etoposide exposure (5 μM, 6 h) in Nalm-6 cells transduced with GFP or scrambled constructs (**Fig. 3B-C**). However, cells transduced with $p21^{WAF1}$ miRNA #2 showed a reduction in basal $p21^{WAF1}$ protein expression (0.9 mean fold change), and complete inhibition of $p21^{WAF1}$ induction after etoposide exposure (**Fig. 3D-E**).

To evaluate the contribution of $p21^{WAF1}$ to drug-induced cell death in ALL cells, transduced Nalm-6 cells were exposed to vorinostat and etoposide, and PS externalization and cell viability were assessed. After 9 h treatment, PS externalization was maximal for etoposide-treated T-ALL PDX cells (**Fig. S4C**). At this time point, $p21^{WAF1}$ knockdown resulted in a significant increase in PS externalization in etoposide and vorinostat treated cells, as evidenced by an increase in the population of early apoptotic (annexin V^+/PI^-) and non-viable (annexin V^+/PI^+) cells (**Fig. 4A-B**, respectively). However, the differences in PS externalization and cell death elicited by $p21^{WAF1}$ knockdown were

Table 1. Biological data and in vitro drug response of ALL PDXs and laboratory established cell lines.

Xenograft/ cell line	ALL subtype	p53 status	$p21^{WAF1}$ status	IC_{50} values (Mean ± SEM, μM)		
				Etoposide	Nutlin-3	Vorinostat
ALL-2	BCP-ALL	wt	Responsive	9.20 ± 1.16	6.49 ± 1.09	7.76 ± 0.71
ALL-3	BCP-ALL	wt	Responsive	0.74 ± 0.05	1.15 ± 0.07	0.57 ± 0.03
ALL-7	BCP-ALL	wt	Responsive	0.64 ± 0.05	1.90 ± 0.51	0.61 ± 0.03
ALL-8	T-ALL	wt	Silenced	0.36 ± 0.01	1.73± 0.53	0.38 ± 0.02
ALL-11	BCP-ALL	wt	Responsive	1.02 ± 0.18	3.23 ± 0.55	1.68 ± 0.23
ALL-16	T-ALL	wt	Silenced	0.31 ± 0.02	1.49 ± 0.04	0.33 ± 0.03
ALL-19	BCP-ALL	wt	Responsive	2.14 ± 0.28	3.63 ± 0.27	2.48 ± 0.34
ALL-27	T-ALL	wt	Silenced	1.22 ± 0.19	5.16 ± 0.89	0.48 ± 0.08
ALL-29	T-ALL	wt	Partial	0.46 ± 0.09	2.06 ± 0.21	0.35 ± 0.03
ALL-30	T-ALL	wt	Silenced	0.54 ± 0.04	1.41 ± 0.22	0.39 ± 0.06
ALL-31	T-ALL	wt	Silenced	1.14 ± 0.11	2.69 ± 0.07	1.94 ± 0.13
Reh	BCP-ALL	wt	Responsive	0.49 ± 0.11	6.65 ± 1.03	2.94 ± 0.46
Nalm-6	BCP-ALL	wt	Responsive	1.44 ± 0.25	4.62 ± 0.31	1.88 ± 0.13
Molt-4	T-ALL	wt	Silenced	0.41 ± 0.03	3.90 ± 0.15	2.52 ± 0.61

$p21^{WAF1}$ status was defined by $p21^{WAF1}$ protein induction after etoposide or vorinostat exposure, as described in Davies *et al*., 2011. Mean IC_{50} values (μM) were determined in this study from 3 independent MTT assays.

not as pronounced following 24 h exposure to etoposide or vorinostat (**Fig. 4C-D**). Moreover, $p21^{WAF1}$ knockdown resulted in only a marginal sensitization of Nalm-6 cells to 48 h of vorinostat and etoposide as assessed by MTT assay. For vorinostat exposure, knockdown of $p21^{WAF1}$ resulted in a statistically significant difference to GFP and scrambled cells in IC_{50} values as determined by one-way ANOVA with multiple comparisons (ANOVA Multiplicity adjusted P-value vs. GFP = 0.023, vs. scrambled = 0.002) (**Fig. 4E-F**, **Table 2**). After etoposide exposure, Nalm-6 cells with $p21^{WAF1}$ knockdown did not demonstrate a significant difference compared to GFP or scramble-transduced cells (ANOVA; Multiplicity adjusted P-value vs. GFP = 0.154, vs. scrambled = 0.117), highlighting that inhibition of $p21^{WAF1}$ expression does not influence sensitivity of cells to p53-inducing agents and re-affirming the results revealed in vorinostat sensitivity for the $p21^{WAF1}$-defective TALL PDXs cells.

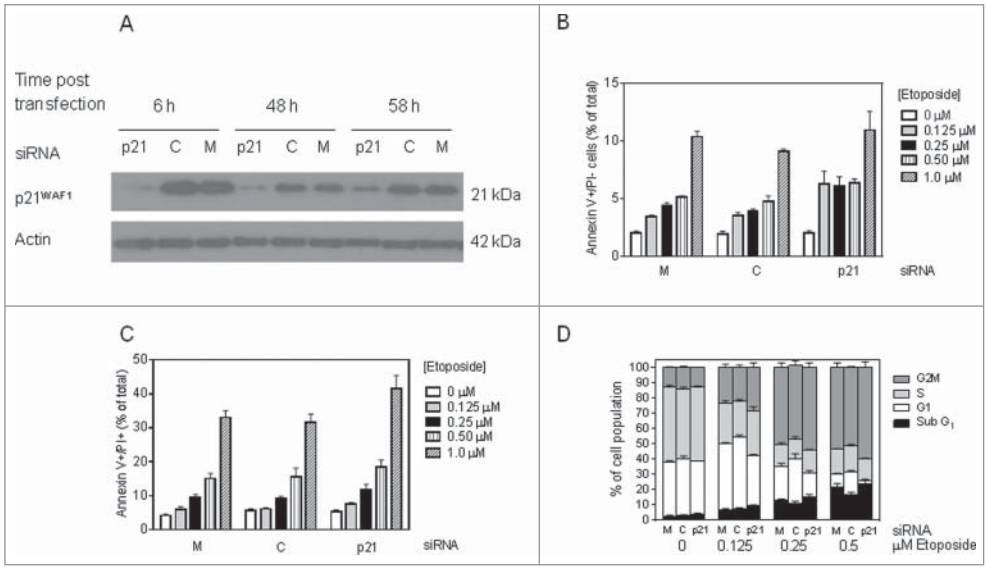

Figure 2. siRNA knockdown of $p21^{WAF1}$ in 697 cells accelerates drug-induced apoptosis. (**A**) $p21^{WAF1}$ expression by western blotting in 697 cells at various time points post siRNA electroporation of $p21^{WAF1}$ siRNA (labeled p21), control siRNA against the MLL-AF4 fusion protein (MLL-AF4 not present in 697 cells) (**C**) and mock transfection (M). (**B-D**) 697 cells were electroporated with the siRNA constructs shown in A, and 16 h later exposed to etoposide (0.125, 0.25 , 0.5, 1 μM) for 48 hr. The proportion of early apoptotic (Annexin V+/PI-), (**B**); non-viable (Annexin V+/PI^+) cells (**C**); and cell cycle distribution, (**D**); were determined by flow cytometry.

Finally, to assess the effects of pharmacological inhibition of $p21^{WAF1}$, the Sp1 inhibitor terameprocol which has previously been demonstrated to inhibit $p21^{WAF1}$ induction, was utilized to inhibit $p21^{WAF1}$ expression after vorinostat and etoposide exposure. Terameprocol inhibited *CDKN1A* mRNA induction after vorinostat exposure in Nalm-6 cells, but had little effect on *CDKN1A* mRNA induction by etoposide (**Fig. 5A**). Consistent with these differential effects on $p21^{WAF1}$ induction by vorinostat and etoposide, terameprocol sensitized Nalm-6 cells to vorinostat to a greater extent than to etoposide (**Fig. 5B**).

Discussion

The anti-apoptotic functions of $p21^{WAF1}$ have been proposed in various models. Yet the influence

of $p21^{WAF1}$ on apoptosis has not been fully characterized in p53-functional hematopoietic cells that are inherently sensitive to cytotoxic stimuli. The investigations contained within evaluated the impact of $p21^{WAF1}$ expression on the apoptotic processes of leukemia cells with a wild-type p53 response to DNA damaging agents and demonstrated a lineage specific response in T-ALL versus BCP-ALL cell death characteristics. As the cell systems tested in these experiments contained functional p53, this data also highlights more broadly the anti-apoptotic role of $p21^{WAF1}$ in p53-functional cells. Previously the two cell lineages demonstrated specific modulation of $p21^{WAF1}$ expression after p53 induction or HDAC inhibition.[23] In the experiments described here, greater caspase-3/7 activity was exhibited by $p21^{WAF1}$-defective T-ALL PDXs after exposure to etoposide and vorinostat in comparison to $p21^{WAF1}$-functional BCP-ALL cells. Maximal caspase activity also occurred earlier in T-ALL cells than in BCP-ALL PDXs, demonstrating increased apoptotic kinetics in T-ALL samples.

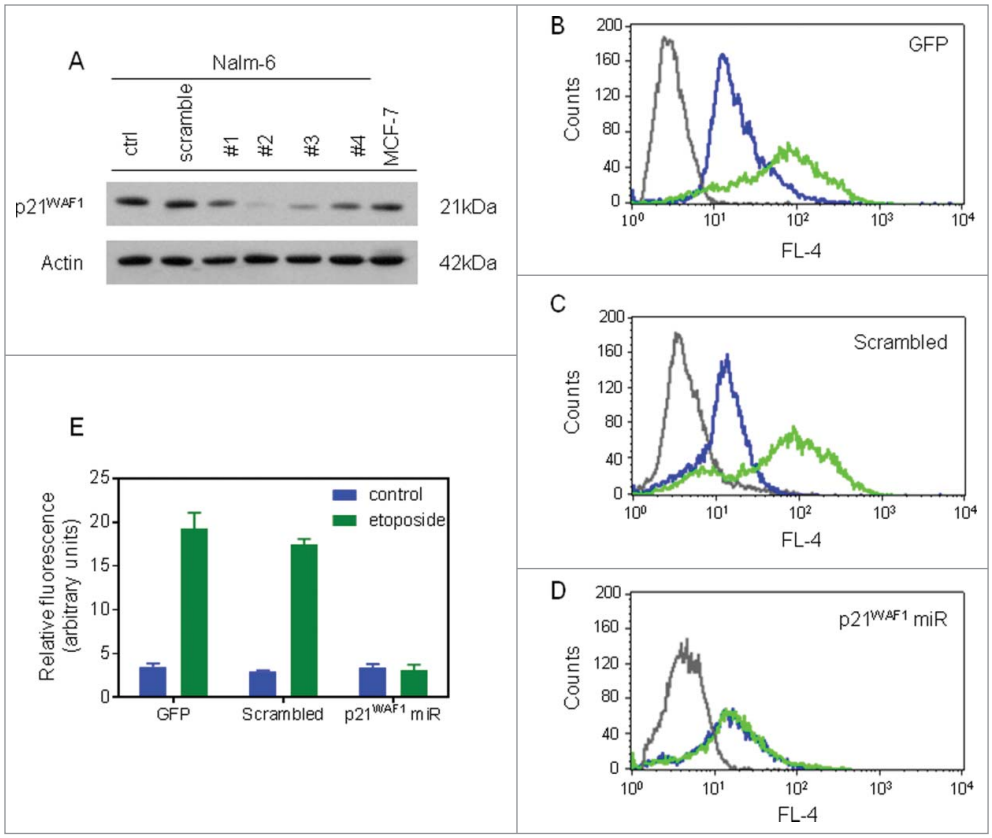

Figure 3. Lentiviral knockdown of $p21^{WAF1}$ in Nalm-6 cells. (**A**) Western blot of lysates prepared from mass-cultures of lentiviral vector-transduced Nalm-6 cells. Numbers represent the different miRNA constructs encoded in the lentiviral constructs. The MCF-7 lysate was included as a positive control for $p21^{WAF1}$ expression. (**B-D**) Transduced Nalm-6 cells were treated with DMSO or etoposide for 6 h and then prepared for detection of $p21^{WAF1}$ expression by dual color flow cytometry. Gray lines, isotype control; blue lines, solvent-treated cells; green lines, etoposide-treated cells. (**B**) GFP or pLent6/V5-EmGFP transduced cells; (**C**) Scrambled or pLenti6/EmGFP-neg-pre-miR (encoding control miRNA) transduced cells; (**D**) $p21^{WAF1}$ miRNA or pLenti6/EmGFP-p21-pre-miRNA #2 ($p21^{WAF1}$ miRNA) transduced cells. (**E**) Graphical representation of the quantified relative fluorescence index of transduced cells with and without etoposide exposure from 3 independent experiments (error bars =mean ± SEM).

PS externalization was another apoptotic characteristic measured in these experiments that also followed a lineage specific trend. PS externalization occurred in T-ALL cells with an intact membrane, yet was only detected in a small proportion of viable BCP-ALL cell samples. The only BCP-lineage cells that exhibited PS externalization comparable to T-ALL after vorinostat and etoposide exposure was the Reh cell line. The Reh cell line was the most sensitive BCP-ALL sample to the effects of etoposide, demonstrated by the lowest IC_{50} value. Although Nalm-6 cells also exhibited PS externalization after drug exposure, this did not occur to the same degree as seen in T-ALL samples.

For T-ALL PDXs, the rate and extent of PS externalization after drug exposure reflected cell sensitivity for this cell lineage. An initial sharp increase in PS externalization on cells with an intact membrane was a characteristic of drug sensitive T-ALL cells, as demonstrated by lower IC_{50} values from MTT assays.

PS externalization has been suggested to be a caspase-dependent feature of apoptosis.[35] Early increase in caspase activity and extensive PS externalization observed in T-ALL PDX complies with this hypothesis. Three BCP-ALL samples showed proportionately less caspase activity and a later time to reach maximal effect, and further demonstrated minimal PS externalization over their untreated controls. An association between high caspase activity and PS externalization was demonstrated in T-ALL PDXs, while high PS externalization was seen in all T-ALL samples (both PDXs and cell lines) tested.

T-ALL cells exhibited increased apoptotic characteristics compared to BCP-ALL when exposed to a range of cytotoxic drugs, despite revealing comparable cell death kinetics. While BCP-ALL cells showed an increase in caspase activity and PS externalization over untreated controls, these results demonstrate that not all forms of cell death necessarily require a robust caspase-3 effect. Caspase activity can be inhibited by a range of cellular processes, such as high expression of Melanoma Inhibitor of Apoptosis Protein, overexpression of survivin, or even by binding of $p21^{WAF1}$ to procaspase-3.[43-45]

T-ALL PDX cells were also sensitive to the death inducing effects of vorinostat, as determined by significantly lower IC_{50}

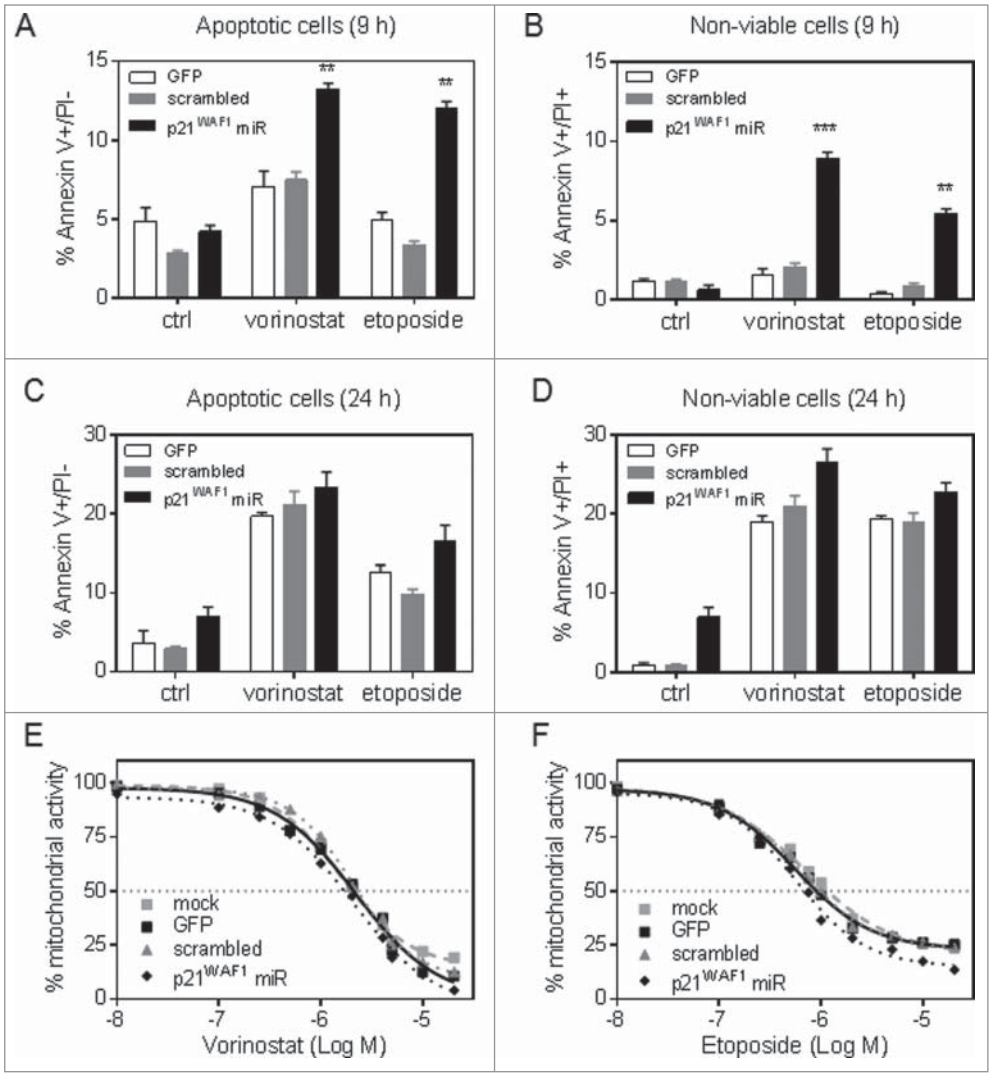

Figure 4. Knockdown of p21WAF1 expression sensitizes Nalm-6 cells to vorinostat- and etoposide-induced apoptosis. Transduced Nalm-6 cells were analyzed for PS externalization and membrane integrity by annexin V binding and PI staining after exposure to vorinostat (5 μM), etoposide (5 μM) or solvent control. (**A**) Early apoptotic (Annexin-V+/PI$^-$PtdIns-) cells at 9 hr. (**B**) Non-viable cells (Annexin V+/PI+) at 9 hr. (**C**) Early apoptotic (Annexin-V+/PI$^-$) cells at 24 hr. (**D**) Non-viable cells (Annexin V+/PI+) at 24 hr. Bars represent the mean ± SEM of 3 experiments. Significantly greater in p21^{WAF1} miRNA transduced cells than GFP and scrambled transduced cells (* = $p<0.05$) by one-way ANOVA with Tukey's multiple comparison test. (**E, F**) Transduced Nalm-6 cells were also treated with vorinostat (**E**) or etoposide (**F**) for 48 h and mitochondrial activity assessed relative to a vehicle treated control using the MTT assay. Results are the mean ± SEM of 3 independent experiments.

values compared to BCP-ALL PDXs. In comparison, the p21^{WAF1}-defective T-ALL and p21^{WAF1}-functional BCP-ALL PDXs demonstrated generally similar sensitivities to the p53-inducing agents, etoposide and nutlin-3. As both T-ALL and BCP-ALL PDXs demonstrated wild-type p53 activity,[23] it would be understandable that both lineages would be equally sensitive to DNA damage and Mdm-2 inhibition. While other perturbations in the p53 pathway besides *TP53* gene mutations do exist, this observation suggests that their impact on the *in vitro* sensitivity of PDX ALL cells would be minimal.

To test whether the defective p21^{WAF1} induction observed in the majority of T-ALL cell samples could have an effect on the apoptotic pathway and their increased sensitivity to the death inducing effects of drugs, p21^{WAF1} expression was suppressed in Nalm-6 cells by transduction with an miRNA construct and by transfection in 697 cells with siRNA. The siRNA-mediated suppression of p21^{WAF1} protein in 697 cells led to an increase in the sub-G1 fraction after etoposide exposure, which has been observed previously with other agents.[10,11,28] Further, this suppression of p21^{WAF1} led to an increase in sensitivity to the death inducing effects of etoposide in 697 cells.

p21^{WAF1}-silenced Nalm-6 cells demonstrated an increase in PS externalization following exposure to vorinostat and etoposide compared to GFP or scramble-transduced cells. Silencing of p21^{WAF1} also increased sensitivity to vorinostat and etoposide, yet between the p21^{WAF1} knockdown cells and either the GFP or scramble-transduced cells, a significant decrease in mean IC_{50} was only seen after vorinostat exposure. Furthermore, greater differences in drug sensitivity were observed between the p21^{WAF1}-defective T-ALL PDXs and the p21^{WAF1}-expressing BCP-ALL cells. Although a weak effect on cell death was noted for p21^{WAF1} in this hematopoietic cell system, other *in vitro* models utilizing different genetic settings or cell types have demonstrated an important role for p21^{WAF1} in inhibiting cell death. A number of studies evaluating the impact of p21^{WAF1} on cell death have been conducted in epithelial derived tumor cells such as MCF-7 or HCT116 cell lines.[6-9,46,47] In general, most of the tumor cell lines showed inherent resistance to the effects of certain cytotoxic stimuli.[7,46] Furthermore, enhanced apoptotic responses of p21^{WAF1}-deficient HCT116 cells to DNA damage occurred after 24 h exposure.[9,46,47] Cell death was observed within 6 h after drug exposure in both T-ALL and BCP-ALL PDXs and cell lines,

Table 2. Impact of p21^{WAF1} repression on the sensitivity of Nalm-6 cells to vorinostat and etoposide.

	IC_{50} values (Mean ± SEM, μM)	
Condition	**Vorinostat**	**Etoposide**
Mock	2.05 ± 0.11	1.51 ± 0.11
GFP-transduced	1.99 ± 0.04	0.91 ± 0.04
Scramble-transduced	2.10 ± 0.06	0.97 ± 0.06
p21^{WAF1}-pre-miR transduced	1.71 ± 0.07[a,b]	0.70 ± 0.07

Statistical analysis of the IC_{50} values generated from each experiment of transduced cells (GFP, Scramble, p21WAF1 miRNA) was conducted by one-way ANOVA with Tukey's multiple comparison test: a = p21^{WAF1} miRNA transduced significant difference compared to GFP transduced cells ($p<0.05$); b = p21^{WAF1} miRNA transduced significant difference to scramble transduced cells ($p<0.005$).

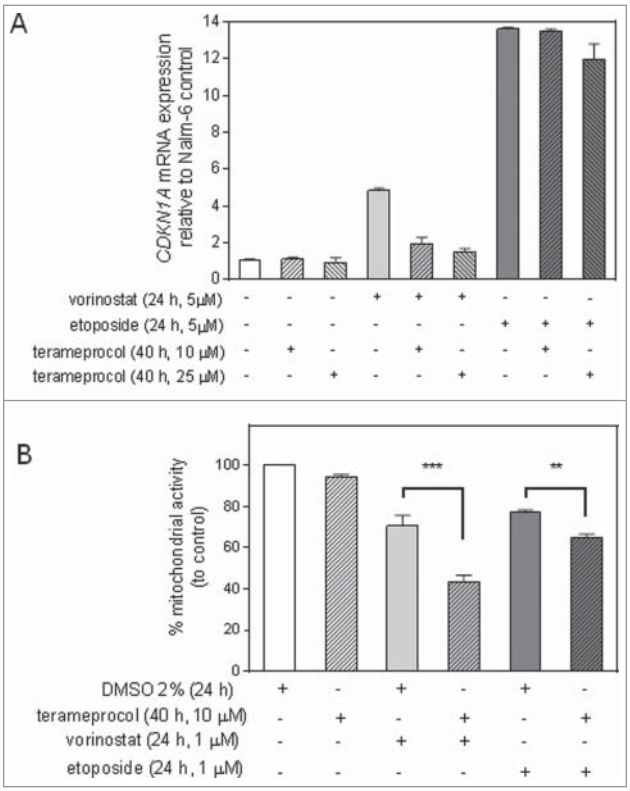

Figure 5. Terameprocol inhibits p21^{WAF1} induction by vorinostat and augments vorinostat-induced cell death in Nalm-6 cells. (**A**) Nalm-6 cells were pre-treated with terameprocol or vehicle control for 16 hr, and then exposed to either etoposide or vorinostat (5 μM, 24 hr). The p21^{WAF1} transcriptional activity was measured by quantitative real time RT-PCR. Bars represent the mean value of *CDKN1A* mRNA ± range of 2 independent experiments. (**B**) Nalm-6 cells were pre-treated with terameprocol or vehicle control for 16 hr, and then exposed to either etoposide or vorinostat (1 μM, 24 hr). Mitochondrial activity was assessed by the MTT cytotoxicity assay and normalized to cells exposed to DMSO solvent. Bars represent the mean % mitochondrial activity of ± SEM of 3 independent experiments. Statistical analysis of the mitochondrial activity after exposure to cytotoxic agents was conducted by one-way ANOVA with Tukey's multiple comparison test and significant differences between vorinostat or etoposide alone and in combination with terameprocol are presented (** = $p<0.05$, *** = $p<0.005$).

demonstrating the increased cytotoxic sensitivity of leukemia cells compared to epithelial derived cell lines.

Only a limited number of studies have reported on the antiapoptotic effect of p21^{WAF1} of hematopoietic cells with many conducted in cells with either a p53-null or mutant genotype.[10,11,13,28] One study used a p53 expressing leukemia cell line (HL-60), and here the disruption of p21^{WAF1} function did not appreciably sensitize the cells to cytarabine.[48] The study herein is the first to report on the anti-apoptotic role of p21^{WAF1} in a BCP-lineage leukemia cell line that was previously shown to have an intact p53 response to DNA damaging agents.[23]

Reports of p21^{WAF1} attenuating apoptosis in myelomonocytic and promyelocytic leukemia cells have demonstrated that inactivation of p21^{WAF1} renders an immediate effect on apoptotic characteristics and drug sensitivity. For example, in the U-937 promyelocytic cell line, increased apoptosis was observed within 4 h of drug exposure.[11] Wang et al.[11] surmised that the mechanism by which p21^{WAF1} dysregulation rendered leukemia cells more sensitive to drugs at earlier time points would differ fundamentally from the mechanisms in colon cancer cells.[7,9,47,49] In our study, p21^{WAF1} silenced Nalm-6 cells demonstrated increased apoptosis, presented in the marker of PS externalization, after 9 h of exposure to etoposide and vorinostat. As silencing of p21^{WAF1} in these cells increased early exposure of PS externalization, this supports the hypothesis that p21^{WAF1} can interact with the initial phase of the apoptotic pathway. As some studies have reported on the interaction of p21^{WAF1} with caspases and have a direct, negative, impact on the apoptotic pathway,[9,50] it is feasible to propose that in the early stages of apoptosis in leukemia cells, caspase activity can also be perturbed by p21^{WAF1}. If p21^{WAF1} is low or not expressed, caspase activity is increased. When p21^{WAF1} is present, cell death still occurs, but with reduced level of apoptotic characteristics. The kinetics of this form of cell death may be slower than classical apoptotic cell death.

In previous studies terameprocol has demonstrated biological activity to inhibit Sp1-mediated gene transcription.[41] HDAC inhibitors such as vorinostat have been shown to induce p21^{WAF1} through Sp1 activation.[51] In these experiments, the selectivity of terameprocol in modulating p21^{WAF1} expression through Sp1 was confirmed as this drug inhibited p21^{WAF1} induction mediated by vorinostat but had no effect on etoposide mediated p21^{WAF1} expression. In combination with vorinostat, terameprocol augmented cell death in leukemia cells that express p21^{WAF1}. These results lead us to propose that attenuation of p21^{WAF1} sensitizes leukemia cells with functional p21^{WAF1} to HDAC inhibitors. In addition to regulating p21^{WAF1} expression, terameprocol can regulate the expression of a range of proteins involved in cell cycle arrest, apoptosis and angiogenesis such as cyclin-dependent kinase 1, survivin and Vascular Endothelial Growth Factor.[52] Therefore, further research with terameprocol should also encompass the influence of these genes to specify the mechanism by which terameprocol can enhance cell death induced by vorinostat. Irrespective of those studies, the results from the present investigations provide proof of the principle that p21^{WAF1} modulation by pharmacological methods may be used to enhance cell death kinetics.

In this study, knockdown of $p21^{WAF1}$ protein sensitized Nalm-6 cells to the apoptotic-inducing effects of vorinostat and etoposide in a time-dependent manner. At early time points, $p21^{WAF1}$ silenced cells exhibited a significant increase in vorinostat and etoposide induced PS externalization, yet at the 24 h time point, there were minimal differences. Chemotherapy-induced cell death still occurred in both T-ALL and BCP-ALL cell populations, demonstrating that high caspase activity was not essential for *in vitro* cell death. The differences in sensitivity observed between the $p21^{WAF1}$-expressing and $p21^{WAF1}$-silenced Nalm-6 cells were not as dramatic as that seen between $p21^{WAF1}$-expressing BCP-ALL cell lines and PDXs and $p21^{WAF1}$-defective T-ALL PDXs cells, highlighting that other characteristics besides differences in $p21^{WAF1}$ induction could contribute to the drug sensitivity demonstrated between ALL cell lineages. The unique genetic characteristics that leukemia cells from different lineages possess could be one of the contributing factors that influence the sensitivity and apoptotic characteristics of the leukemia cell lineages. Previous work with T-ALL and BCP-ALL PDXs have established distinct gene expression profiles for the 2 leukemia lineages,[53] while a subset-of the T-ALL and BCP-ALL PDXs tested have demonstrated differential expression of certain p53-target genes in response to etoposide.[23] A group of genes that have been demonstrated to influence the apoptotic response to cytotoxic drugs is the Bcl-2 gene family. The basal expression levels of pro-apoptotic or anti-apoptotic Bcl-2 genes were presented for T-ALL and BCP-ALL PDXs in a recent publication.[53] These results did not show a distinct homogeneous pattern of expression within lineages for individual members of this gene family and therefore basal gene expression was unable to explain simply the cell lineage differences in apoptotic response and drug sensitivity.

While the differences between T-ALL and BCP-ALL PDX sensitivity to vorinostat *in vitro* were modest, when previously tested *in vivo* by our group vorinostat was universally ineffective against the same pediatric ALL PDXs used in this study.[54] However, the second-generation inhibitor of Class I and II HDACs, quisinostat (JNJ-26481585), exerted profoundly increased *in vivo* anti-leukemic efficacy against 2 T-ALL PDXs (ALL-8 and -16) compared with 6 BCP-ALL PDXs (including ALL-2, -3, -7, -11 and -19).[55] Therefore, epigenetic silencing of $p21^{WAF1}$ may provide a biomarker for the enhanced *in vivo* sensitivity of T-ALL to second-generation HDAC inhibitors.

Patients, Materials and Methods

All experimental studies were conducted with approval from the Human Research Ethics Committee and the Animal Care and Ethics Committee of the University of New South Wales (Sydney, Australia).

In vitro cell culture

Leukemia cell lines [697 (or PreB-697), Reh, Nalm-6, Molt-4] were obtained from the American Type Culture Collection (Manassas, VA, USA) or the European Collection of Cell Cultures (Salisbury, UK), and maintained in Roswell Park Memorial Institute (RPMI-1640) media supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 μg/mL), and L-glutamine (2 mM). 293FT cells were purchased from Invitrogen (Catalog Number (#) R700-07) and maintained in Dulbecco's Modified Eagle media (DMEM) with the same supplements. PDX cells were prepared for *in vitro* culture as described previously.[56] Lineage subtype and other details of PDXs and cell lines are shown in **Table 1**.

Patient clinical samples

Leukemia cells were obtained from the Centre for Children's Cancer and Blood Disorders at Sydney Children's Hospital from children presenting with ALL that were enrolled in Australia and New Zealand Children's Cancer Study Group (ANZCCSG) Study VII (1998-2001) or Study VIII (2002 to present). Protocols for processing samples, harvesting, cryopreservation and establishing PDX cells are detailed in previous work.[57]

In vitro cytotoxicity assays

In vitro drug sensitivity was assessed using the colorimetric methyl-thiazolyl-tetrazolium (MTT) assay, and is described elsewhere.[56] The cells were exposed to etoposide (Sigma-Aldrich, #E1383), vorinostat (donation from Merck Research Laboratories), terameprocol (donation from Erimos Pharmaceuticals) or an equivalent volume of dimethyl sulfoxide (DMSO) vehicle (Sigma-Aldrich).

Caspase activity assays

Caspase activity was measured using the Caspase-3 Colorimetric Assay (Sigma-Aldrich, #CASP3C) following the manufacturer's instructions. Vorinostat (5 μM), etoposide (5 μM) or staurosporine (1 μM) or an equivalent volume of DMSO were added to cells for 0, 6, 16 and 24 h. Mean absorbance at A_{405nm} on a Biotrack II spectrophotometer plate reader (Amersham Biosciences, Piscataway, NJ, USA) from triplicate samples was compared to a series of pnitroaniline (pNA) standard concentrations, and caspase activity expressed as an amount of Ac-DEVD-pNA released.

PS externalization assays

PS translocation from the inner surface of the plasma membrane to the outer leaflet was detected using Annexin V binding assay. PDX cells and ALL cell lines were treated over 48 h with cytotoxic stimuli (5 μM etoposide, 5 μM vorinostat, 1 μM dexamethasone, 1 μM staurosporine) or an equivalent volume of DMSO. At predetermined time points, the cells were washed with phosphate buffered saline (PBS), then incubated with 5 μL annexin V solution (FITC or APC, BD PharMingen, San Diego, CA, USA) and 250 ng propidium iodide (PI) in 200 μL of Annexin-V binding buffer (BD PharMingen, #556454). Cells were analyzed and data collected on a BD FacsCanto flow cytometer (BD PharMingen San Diego, CA, USA) using CellQuest software. 697 cells were treated over 24 h with etoposide (0.125-1 μM). Cells (3×10^5) were then washed twice in PBS followed by suspension in 250 μL of binding buffer containing 125 ng

Annexin V (Abcam, #ab14082) and 100 ng PI. Cells were incubated for 20 min and a further 250 μL of binding buffer was then added prior to analysis on a BD FACScan flow cytometer (BD PharMingen) using CellQuest software.

Cell cycle analysis

697 cells were treated over 24 h with etoposide (0.125-1 μM). Cells (3 x 10^5) were re-suspended in 100 μL of 0.25 M sucrose, 40 mM sodium citrate (pH 7.6), 400 μL of DNA staining buffer (20 μg/mL PI, 0.5% NP-40 and 0.5 mM EDTA in PBS) and 28.8 μL of 44 mg/mL DNase free RNase A. Samples were incubated at 4°C in the dark for 30 min prior to analysis with BD FACScan cytometer (BD PharMingen) using CellQuest software.

Quantitative RT-PCR

RNA extraction and real-time quantitative reverse transcription polymerase chain reaction (RT^2-PCR) was carried out as previously described.[56] Primers and probes for *CDKN1A* (*$p21^{WAF1}$* gene) were purchased from Applied Biosystems (Hs00355782_m1). The Elongation factor-1a (*EF-1*α) gene was an internal standard in each reaction (primers EF-1αF, 5′-CTGAACCATCCAGGCCAAAT-3′; EF-1αR, 5′-GCCGTG TGGCAATCCAAT-3′; Probe, 5′-VIC-AGCGCCGGCTATG CCCCTG-TAMRA-3′). RNA levels were normalized to EF-1α values, expressed as fold differences relative to *CDKN1A* mRNA expression in Nalm-6 cells.

siRNA transfection

The 697 cell line was transfected with siRNA construct p21 SHS1.Hs01.00025255 (Sigma-Aldrich) or control siRNA construct siMA6 against the MLL-AF4 fusion, not present in 697 cells (sense construct 5′-AAGAAAAGCAGACCUACUCCA-3′) (Invitrogen). Cells (790 μL of 1 x 10^7/mL) were placed into an electroporation cuvette and 10 μL of either 10 μM $p21^{WAF1}$ siRNA, 10 μM control (siMA6) siRNA (final siRNA concentration 250 nM) or water (mock control) were added and electroporated at 350V, 1200μF, 10ms with an EPI 2500 Electroporation Pulse Generator and then left for 15 min before diluting in 9 mL of culture media. Cells were incubated at 37°C, 5% CO_2 for 16 h, from siRNA transfection, prior to addition of drug.

Lentiviral transductions

CDKN1A gene ($p21^{WAF1}$) knockdown was performed using pLenti6/EmGFP lentiviral constructs (Invitrogen, #V496-10) in Nalm-6 cells. DNA oligonucleotides were designed using the RNAi Designer software available from Invitrogen to encode a target pre-microRNA (pre-miRNA) for *CDKN1A* as described in the BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector instruction manual. Briefly, 4 double-stranded oligonucleotides encoding the engineered pre-miRNA were prepared having, among other structural features, a 5′G + short 21 nucleotide antisense sequence (mature miRNA) targeting the *CDKN1A* gene followed by 19 nucleotides to form the terminal loop and a short sense target sequence with 2 nucleotides removed to create an internal loop. The double-stranded DNA oligonucleotides were prepared from the set of single stranded oligonucleotides (**Table S1**) by PCR, annealed and ligated into an intermediary pcDNA plasmid construct. Lentiviral vectors encoding the green fluorescence protein (GFP) and a $p21^{WAF1}$ pre-miRNA cassette driven by a CMV promoter were constructed utilizing Gateway→ recombination technology from the pcDNA plasmid constructs to produce pLenti6/EmGFP $p21^{WAF1}$ microRNA vector. DNA was isolated from bacterial cultures using Purelink HQ Mini Plasmid Purification kit (Invitrogen, #K2100-15). Sequencing of plasmid DNA was conducted at Sydney University Prince Alfred Molecular Analysis Center (University of Sydney, NSW). Plasmids and PCR products were digested with restriction enzymes; *Bam*HI and *Hind*III (Promega, #R6021 and #R6041) were used to linearize pcDNA constructs for verification, while *Eag*I (New England Biolabs Inc., #R0505S) was used to linearize pcDNA6.2-GW/EmGFP-p21miR constructs before cloning.

Lentiviral infections were carried according to standard procedures for silencing experiments. 293FT cells were transfected using Lipofectamine (Invitrogen, #11668-019) as per the manufacturer's protocol with Virapower Packaging mix (Invitrogen, #K4975-00) and 3 μg of lentiviral plasmid in 3 mL Opti-MEM containing 10% FCS. The next day, media containing the DNA-Lipofectamine complexes was removed and replaced with 10 mL of complete DME media without antibiotics. The virus-containing media (VCM) was harvested from the 293FT cells after 24 h, and replaced with fresh complete DME media. VCM was centrifuged at 490 *x* g for 15 min at 4°C to pellet debris and filtered through a 0.45 μm filter. Thereafter, every 12 h, VCM was harvested from 293FT cells for infection of the Nalm-6 leukemia cell line.

Nalm-6 cells (2 x 10^5) were transduced with VCM prepared from pLenti6/EmGFP $p21^{WAF1}$ miRNA vector ($p21^{WAF1}$ miRNA), pLenti6/EmGFP vector (GFP-transduced) or pLenti6/EmGFP-scrambled vector (scramble) on 3 consecutive days at a Multiplicity of Infection (MOI) of 30 in a minimal volume of media with polybrene (8 μg/mL). After incubation in VCM for 72 h, Nalm-6 cells were expanded in complete RPMI media.

Analysis of protein expression

The preparation and separation of whole cell lysates from PDX cells and leukemia cell lines (Nalm6 and 697) have been described previously.[23,56] 697 cell line lysates were prepared using lysis buffer supplied by Cell Signaling Technology (New England Biolabs, #9803), containing a protease inhibitor cocktail (Roche, UK). Membranes were probed with mouse antibodies for $p21^{WAF1}$ (clone SX118BD: BD Transduction Laboratories, #556430 or clone 70: BD Biosciences, #610234) and rabbit or mouse antibodies for actin (Sigma-Aldrich #A3853 or Clone JLA20, Calbiochem, #MABT219), followed by horseradish peroxidase (HRP) conjugated donkey anti-rabbit, sheep anti–mouse secondary antibodies (GE Healthcare, #RPN4301 and #RPN4201), or goat anti-mouse or anti-rabbit secondary antibodies (Dako, #P044701-2 and #P044801-2). Bound secondary antibodies were detected by chemiluminescence and visualized by autoradiography detection and phosphoimaging as described previously.[23]

Cellular $p21^{WAF1}$ protein expression was measured in Nalm-6 cells after lentiviral transduction by flow cytometry. Following treatment with etoposide (5 μM, 6 h), transduced cells were fixed in 1% formaldehyde in PBS for 15 min, washed in PBS and permeabilized with 200 μL 0.1% Triton X-100. Fixed cells were incubated with anti-human $p21^{WAF1}$ antibody (Clone SX118), and then a secondary anti-mouse IgG Cy5-conjugated antibody (Invitrogen, #A10524).

Statistical Comparison and Data Analysis

All data were compiled, the mean and standard error of mean (SEM) of data sets calculated using GraphPad Prism software (version 6.02). Mann-Whitney *U* tests (for non-normally distributed data) were utilized to compare differences between groups. Significance level was set at $p < 0.05$. Analysis of multiple samples was conducted by one-way ANOVA with Tukey's multiple comparison test to examine results between groups.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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