Effective Targeting of the P53-MDM2 Axis in Preclinical Models of Infant MLL-Rearranged Acute Lymphoblastic Leukemia

Jennifer Richmond1, Hernan Carol1, Kathryn Evans1, Laura High1, Agnes Mendomo1, Alissa Robbins1, Claus Meyer2, Nicola C. Venn1, Rolf Marschalek2, Michelle Henderson1, Rosemary Sutton1, Raushan T. Kurmasheva3, Ursula R. Kees4, Peter J. Houghton3, Malcolm A. Smith5, and Richard B. Lock1

Abstract

**Purpose:** Although the overall cure rate for pediatric acute lymphoblastic leukemia (ALL) approaches 90%, infants with ALL harboring translocations in the mixed-lineage leukemia (MLL) oncogene (infant MLL-ALL) experience shorter remission duration and lower survival rates (~50%). Mutations in the p53 tumor-suppressor gene are uncommon in infant MLL-ALL, and drugs that release p53 from inhibitory mechanisms may be beneficial. The purpose of this study was to assess the efficacy of the orally available nutlin, RG7112, against patient-derived MLL-ALL xenografts.

**Experimental Design:** Eight MLL-ALL patient-derived xenografts were established in immune-deficient mice, and their molecular features compared with B-lineage ALL and T-ALL xenografts. The sensitivity of MLL-ALL xenografts to RG7112 was assessed in vitro and in vivo, and the ability of RG7112 to induce p53, cell-cycle arrest, and apoptosis in vivo was evaluated.

**Results:** Gene-expression analysis revealed that MLL-ALL, B-lineage ALL, and T-ALL xenografts clustered according to subtype. Moreover, genes previously reported to be upregulated in MLL-ALL, including MEIS1, CCNA1, and members of the HOXA family, were significantly upregulated in MLL-ALL xenografts, confirming their ability to recapitulate the clinical disease. Exposure of MLL-ALL xenografts to RG7112 in vitro caused p53 upregulation, cell-cycle arrest, and apoptosis. RG7112 as a single agent induced significant regressions in infant MLL-ALL xenografts. Therapeutic enhancement was observed when RG7112 was assessed using combination treatment with an induction-type regimen (vincristine/dexamethasone/L-asparaginase) against an MLL-ALL xenograft.

**Conclusion:** The utility of targeting the p53-MDM2 axis in combination with established drugs for the management of infant MLL-ALL warrants further investigation. Clin Cancer Res; 1–11. ©2015 AACR.

Introduction

Overall cure rates for pediatric acute lymphoblastic leukemia (ALL) have improved considerably in the last 50 years due to improvements in the use of multiagent chemotherapy and advances in supportive care, such that almost 90% of patients now experience long-term survival (1, 2). Despite this success, subsets of patients are associated with a poor prognosis. Infants (<12 months of age) diagnosed with ALL frequently present with a range of high-risk features, including high leukocyte count at diagnosis, an immature CD10-negative phenotype, and coexpression of myeloid antigens. However, the most distinctive genetic feature of infant ALL is the presence of rearrangements involving the MLL (mixed-lineage leukemia) oncogene at the 11q23 chromosomal region (3–5). MLL translocations are found in nearly 80% of infants diagnosed with ALL compared with 2% to 4% of older children, and confer a poorer prognosis than for infants with germline MLL (6–8).

Between 90% and 95% of infants with ALL achieve remission following intensive induction therapy using established drugs, including glucocorticoids, vincristine, L-asparaginase, cytarabine, daunorubicin, and methotrexate (6). However, despite this intensive treatment there is a high rate of relapse, and the overall survival rate for infants with ALL harboring an MLL rearrangement is approximately 50% (9, 10). This is in part due to the fact that the patients with confirmed MLL translocations are often particularly resistant to glucocorticoids such as prednisone and dexamethasone, which are key components in current ALL chemotherapy treatments (6, 11, 12). Studies in vitro have also shown that MLL-ALL has a distinct drug resistance profile in comparison with childhood ALL, with high levels of resistance to glucocorticoids and L-asparaginase observed (13). These results highlight the need for treatment protocols that are more specifically tailored...
for MLL-ALL and the need for targeted therapies that could be incorporated to strengthen current combination chemotherapy regimens.

The p53 tumor suppressor has long been an attractive therapeutic target for anticancer strategies. Once p53 is activated in response to cellular stress it initiates the transcription of p53-related genes that are involved in cell-cycle arrest, senescence, and apoptosis, thereby preventing the proliferation of genetically unstable cells in its function as a key suppressor of tumorigenesis (14). Because errant activation of p53 could have disastrous consequences for multicellular organisms, it is tightly regulated primarily through its interaction with the ubiquitin E3 ligase MDM2 (mouse double minute 2), which suppresses p53 transcriptional activity and promotes its proteasomal degradation (15–17). It is estimated that p53 mutations are present in approximately 50% of all human cancers (14). However, they are relatively infrequent in pediatric ALL, being detected in approximately 2% and 6% to 2% of diagnosis and relapse cases, respectively (18–20). Although p53 mutations may be less prevalent in pediatric cancer, loss of p53 function is characteristic of virtually all cancers as even those that retain wild-type p53 use alternative mechanisms to impede its function (21). One such mechanism is the over expression of MDM2 (22), present in 20% to 30% of patients with ALL, and is often associated with chemoresistance and a poor prognosis (23–25).

Within the past decade, several strategies have been developed to reactivate p53 function in hematologic malignancies, including targeting the MDM2–p53 interaction (26–30). RG7112 is an orally available ci-isimidazole that binds to the hydrophobic pocket of MDM2, blocking its interaction with p53 resulting in p53-mediated induction of apoptosis (31, 32). RG7112 was previously reported by the Pediatric Preclinical Testing Program to exhibit broad antileukemic efficacy against a heterogeneous panel of patient-derived xenografts, with enhanced activity against a single infant MLL-ALL xenograft tested (31). Gene-expression analysis revealed that the ALL panel had relatively high basal levels of p53 compared with other pediatric cancer histotypes, indicating that pediatric ALL may be primed for unleashing p53 from MDM2 inhibition to initiate apoptosis. Because of the relatively poor outcome for infant MLL-ALL, our preliminary report of significant in vivo RG7112 efficacy against a single infant MLL-ALL xenograft (31) clearly warranted additional evaluation against a larger panel of infant MLL-ALL patient-derived xenografts. We now report the molecular characterization of a panel of patient-derived infant MLL-ALL xenografts, their in vivo responses to single-agent RG7112, and the ability of RG7112 to exert therapeutic synergy with an induction-type regimen of vincristine, dexamethasone, and L-asparaginase (VXL).

Materials and Methods

Development of an infant MLL-ALL xenograft panel

All studies had prior approval from the Human Research Ethics Committees of the University of New South Wales and the Dana-Farber Cancer Institute, and the Animal Care and Ethics Committee of the University of New South Wales. Human leukemia cells used in this study were from peripheral blood or bone marrow biopsy specimens of infants with MLL-ALL and were kindly provided by Dr. Scott Armstrong (Dana-Farber Cancer Institute, Boston, MA). Detailed methods used to establish patient-derived xenografts from pediatric ALL biopsies in NOD/SCID (NOD.CB17-Prkdcsid/J) mice have been previously described (33). Briefly, leukemia cells from patients were inoculated i.v. into female 20- to 25-g 6- to 8-week-old NOD/SCID mice (Australian BioResources). Leukemia engraftment was monitored by flow cytometric quantification of the proportion of human CD45+–positive (huCD45+) cells versus total CD45+ leukocytes (human + murine) in the peripheral blood and tissues as described previously (33, 34). The rate of engraftment was measured as the time in days following transplantation for leukemia cells to disseminate and reach 5% huCD45+ in peripheral blood. To establish continuous xenografts spleen-derived cells (usually at >95% huCD45+ purity) from engrafted mice were inoculated into secondary and tertiary recipient mice. MLL translocations were confirmed by long distance inverse-PCR as previously described (35), and serial passage xenografts were validated using an SNP array assay.

Microarray analysis of gene expression

Gene-expression profiling on RNA extracted from spleen-derived cells was performed using the Illumina Human Ref-12 Expression BeadChip (Illumina Inc.). The sample gene profiles obtained were normalized using quantile normalization and log transformed using GenomeStudio (Version 1.6.0, Illumina Inc.). Differential gene expression was established using limma, based on a moderate t-statistic. After analysis in limma, the FDR was used to adjust the P value for multiple testing. In this study, differences in gene expression were deemed significant with an FDR ≤ 0.05. All statistical analyses and heatmaps were generated using GenePattern (36). Gene-expression datasets can be accessed at www.ncbi.nlm.nih.gov/geo (accession no. GSE52991).

In vitro cell culture and cytotoxicity assays

RS4;11, Jurkat, CEM, NALM6 cell lines were all obtained from commercial suppliers and used within 3 months of culture following validation by short tandem repeat (STR) analysis. Cell lines were maintained in RPMI supplemented with 10% heat-inactivated FCS, 100 U/mL penicillin, 100 μg/mL streptomycin,
and 2 mmol/L-glutamine (Life Technologies). ALL cell lines were validated by STR analysis, verified mycoplasma-free, and cultured for <3 months. Xenograft cells were retrieved from cryostorage and resuspended in QBSF-60 medium (Quality Biological) supplemented with 20 ng/mL Fli-3 ligand, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L-glutamine. Before treatment, cells were plated in 96-well plates (100 μL/well) at a density previously optimized and were equilibrated overnight at 37°C with 5% CO2. Cells were then treated with 10-fold serial dilutions of RG7112 (10 μmol/L–1 pmol/L) for 48 hours, at which point AlamarBlue reagent [0.6 mmol/L Resazurin, 0.07 mmol/L Methylene Blue, 1 mmol/L potassium hexacyanoferrate (II), 1 mmol/L potassium hexacyanoferrate (II) trihydrate] was added. Fluorescence was measured at 0 and 6 hours following addition of AlamarBlue using a fluorescent plate reader (VICTOR3 PerkinElmer) with excitation at 560 nm and emission at 590 nm. Data are expressed as a percentage of untreated controls.

Combination cytotoxicity assays
For fixed ratio combination assays, cells were treated with RG7112 and vincristine, dexamethasone, or L-asparaginase at 0.25, 0.5, 1, 2, and 4 times the relevant IC50 values for each cell line/xenograft. Drug effects were assessed by AlamarBlue as described in the Materials and Methods, and combination indices (CI) were calculated using CalcuSyn software (Version 2.0; Biosoft) to determine possible drug synergies. CI values <0.9 and >1.1 were considered synergistic and antagonistic, respectively.

Cell-cycle and apoptosis assays
RS4:11 cells were seeded in 96-well plates (10⁵ cells/mL) and incubated with 5 μmol/L RG7112 for up to 72 hours, and 10 μmol/L of EdU (5-ethynyl-2′-deoxyuridine; Life Technologies) was included in some experiments. Apoptosis was determined using Annexin V/7-AAD staining, and cell-cycle analysis was assessed using the Click-iT EdU cell proliferation assay according to the manufacturer’s instructions (Life Technologies). Samples were run on a FACScan and analyzed using FlowJo (version X.0.6) software (Tree Star Inc.).

Protein expression analysis
Methods for preparation of whole-cell extracts, determination of protein concentrations, and analysis of cellular proteins by immunoblotting have been described in detail previously (37). Membranes were probed with specific antibodies for the following proteins: p53 (sc-126), MDM2 (SMP14), p21WAF1 (sc-817; Santa Cruz Biotechnology), PUMA (D30C10; Cell Signaling Technology), and actin (Sigma-Aldrich). The secondary antibody used was horseradish peroxidase (HRP) conjugates of anti-rabbit IgG (GE Healthcare). Signals were detected by Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore) and visualized using VersaDoc 5000 Imaging System (Bio-Rad).

In vivo drug treatments
MLL-ALL xenograft cells were transplanted into 20- to 25-g 6- to 8-week-old female NOD/SCID mice by i.v. injection. When the median percentage of huCD45⁺ cells in the peripheral blood was above 1% for the entire cohort, mice were randomized and allocated to treatment groups (6–9 mice/group). RG7112 was supplied, preformulated by Roche Pharmaceuticals Inc. through the Cancer Therapy Evaluation Program (NCI). For single-agent efficacy studies, RG7112 was administered orally (per os; P.O.) at a dose of 100 mg/kg daily for 14 days. For combination studies, RG7112 was given daily for 5 days at 100 mg/kg (P.O.). VXL (Prince of Wales Hospital Pharmacy, NSW, Australia) was administered i.v. p. injection over the same 5-day period, consisting of vincristine (0.15 mg/kg) on day 0 followed by daily dexamethasone (5 mg/kg) and L-asparaginase (1,000 U/kg; ref. 38). Because of side effects observed during a prior tolerability experiment, standard mouse chow was replaced with a nutrient fortified water gel [DietGel recovery (Techniplast)] for the duration of the combination treatment.

Determination of in vivo treatment response
Individual mouse event-free survival (EFS) was calculated as the time in days from treatment initiation until the percentage of huCD45⁺ cells in the peripheral blood reached 25%, or until mice reached a humane end-point with evidence of leukemia-related morbidity. EFS time was represented graphically by Kaplan–Meier analysis and survival curves were compared by the log-rank test. The response to drug treatment was evaluated by two methods: (i) leukemia growth delay (LGD), calculated as the difference between the median EFS of the drug-treated cohort and the median EFS of the vehicle-treated cohort; and (ii) using an objective response measure (ORM) modeled after stringent clinical criteria, which was assessed at day 42 posttreatment initiation as previously described (39) and detailed in the Supplementary Methods.

In vivo pharmacodynamic analysis
Mice were inoculated with MLL-ALL xenograft cells and monitored until peripheral blood engraftment reached >70% huCD45⁺, at which stage a single dose of RG7112 (100 mg/kg) was administered. Groups of 3 mice were culled and spleens harvested at each time point of 6, 12, and 24 hours posttreatment. Protein expression was analyzed by immunoblotting and cell-cycle status by flow cytometry. For EdU cell-cycle analysis, 1.25 mg was administered i.v. injection to mice 2 hours before sample collection.

Statistical analysis
The exact log-rank test as implemented using Proc Stat-Xact for SAS was used to compare EFS distributions between treatment and control groups (two-tailed), with a P value of ≤0.05 considered significant. To evaluate interactions between drugs in vivo, therapeutic enhancement was considered if the EFS of mice treated with the combination of RG7112 and VXL was significantly greater (P < 0.01) than those induced by both single arms of the study (40, 41).

Results
Development and characterization of a panel of MLL-rearranged xenografts
The engraftment and characterization of a panel of ALL primary patient biopsies in immune-deficient NOD/SCID mice has been previously described (33, 34). To expand the ALL xenograft panel to include other high-risk subtypes, eight patient biopsies were obtained from infants diagnosed with MLL-ALL and inoculated
into NOD/SCID mice. Seven of these showed robust evidence of engraftment and dissemination into the peripheral blood, whereas another caused hind-limb paralysis and was not used in subsequent experiments. The engraftment rates of these MLL-ALL xenografts over three passages are detailed in Supplementary Fig. S1A and Supplementary Table S1. Three xenografts showed no significant changes in engraftment rates at 2nd and 3rd passage compared with 1st passage (MLL-3, -6 and -8); two showed acceleration at 2nd and 3rd passage (MLL-5 and -7); one accelerated only at 3rd passage (MLL-2); and one showed variable rates of engraftment at 2nd and 3rd passage (MLL-1). At first passage all xenografts showed high-level infiltration of bone marrow and spleen (Supplementary Fig. S1B), with variable infiltration of peripheral organs consistent with the clinical experience in this disease (42). In addition, one MLL-ALL xenograft (MLL-14) with a t(11;19) (MLL-ENL translocation) that had previously been established in our laboratory was also used in this study (43). MLL translocations were confirmed by long distance inverse-PCR and are detailed in Supplementary Table S1.

Previous studies have clearly demonstrated that MLL translocations specify a gene-expression profile that is distinct from other ALL subtypes, including B-cell precursor ALL (BCP-ALL) and acute myelogenous leukemia (4, 44). Unsupervised hierarchical clustering revealed that a large xenograft panel clustered according to ALL subtype (Fig. 1A), indicating that the genes that differentiated each leukemia subtype were conserved through serial xenografting. The BCP-ALL xenograft ALL-3 clustered with the MLL-ALL xenografts, in accordance with it harboring a t(11;19) and MLL-ENL translocation (33, 34). Also, MLL-ALL xenografts loosely clustered according to their specific MLL translocation (Fig. 1B; Supplementary Table S1). Of the approximate 34,600 genes tested, 1,419 were upregulated and 1,235 downregulated in the nine MLL-ALL xenografts compared with 17 BCP-ALL xenografts (defined by FDR < 0.05). The top 100 genes that were most highly correlated with each class distinction are shown in Fig. 1C; additional information on these genes, including FDR values, is detailed in Supplementary Table S2. Genes previously reported (4, 11, 44) to be underexpressed in MLL-translocated versus nontranslocated leukemia [including MME (CD10), LARGE, ID3 and ALOX5] are represented in the list (Fig. 1C; Supplementary Fig. S2A and Supplementary Table S2). Genes reported to be overexpressed in MLL-translocated leukemia, including MEIS1 (a cofactor of HOXA9 and HOXA7), RNASE3 and IGF2BP2, were also significantly upregulated in the MLL-ALL xenografts (Fig. 1C; Supplementary Fig. S2B and Supplementary Table S2). Moreover, the HOXA genes, which are reported to be overexpressed in the majority of infant MLL-ALL cases (4, 45, 46), were overexpressed in six of nine MLL-ALL xenografts (Supplementary Fig. S3). The differences in expression of all of these HOXA genes were significant between the MLL-ALL and BCP-ALL xenografts (Supplementary Fig. S3B).

We then asked whether the unique gene-expression signature for MLL-translocated ALL reported by Armstrong and colleagues (4) was represented in the MLL-ALL xenografts that had undergone serial passageing in vivo. The Armstrong signature clearly separated the MLL-ALL and BCP-ALL xenografts (Fig. 1D) with 70% of the genes identified by Armstrong and colleagues being significantly differentially expressed between the two xenograft panels (Fig. 1D with statistical analysis shown in Supplementary Table S3). Collectively, these results confirm that the unique gene-expression signature distinguishing MLL-translocated leukemia is maintained during serial passage through NOD/SCID mice.

RG7112 activates p53 and induces cell-cycle arrest and apoptosis in leukemia cells in vitro

In preparation for assessing the efficacy of RG7112 against the panel of MLL-ALL xenografts described above, we first tested its in vitro cytotoxicity against leukemia cell lines of known p53 status. Consistent with the literature (32), the cytotoxic effects of RG7112 appeared to be dependent on p53 status; NALM6 and RS4;11 (p53 wild-type) exhibited IC₅₀ of 3.2 and 1.4 μmol/L, respectively, whereas those of JURKAT and CEM (p53 mutant) were >10 μmol/L (Fig. 2A). When assessed using the same in vitro assay MLL-ALL xenograft cells exhibited IC₅₀ of 0.1 to 0.35 μmol/L, consistent with wild-type p53 status (Fig. 2A). The wild-type p53 status of the infant MLL-ALL xenografts was also confirmed by exon sequencing (data not shown).

To define the mechanism of RG7112-induced cell death of MLL-ALL cells, we next showed that exposure of RS4;11 cells to RG7112 resulted in profound upregulation of the p53 protein within 6 hours, as well as its downstream targets p21WAF1 and PUMA (Fig. 2B). This activation of p53 was associated with a profound decrease in cells in S-phase, and accumulation of cells in G1 (Fig. 2C). This cell-cycle arrest was followed by induction of apoptosis, as assessed by Annexin V/7AAD staining (Fig. 2D and Supplementary Fig. S4), and cleavage of caspase-3 (Fig. 2B). Because the IC₅₀ values of the MLL-ALL xenograft cells were approximately 10-fold lower than RS4;11 cells, we next exposed MLL-5 and MLL-14 xenograft cells to a 10-fold lower concentration of RG7112 than RS4;11 cells in vitro, and observed similar findings. Immunoblot analysis revealed marked upregulation of p53 within 6 hours, as well as the downstream proteins p21WAF1, PUMA, and cleaved caspase-3 in both xenografts (Fig. 2B).

Because RG7112 has been previously reported to induce apoptosis in a caspase-independent manner (32), we treated RS4;11 cells with RG7112 in the presence of the pan-caspase inhibitor QVD. QVD alone had no effect on the viability of RS4;11 cells for up to 72 hours (Fig. 2D and Supplementary Fig. S4). However, it substantially delayed and inhibited RG7112-induced cell death via apoptosis, as measured by its effects on RG7112-induced loss of cell viability and its ability to prevent cleavage of caspase-3 (Fig. 2B). In contrast, QVD did not inhibit the ability of RG7112 to induce p53, p21WAF1 or PUMA (Fig. 2B), and cell-cycle arrest (Fig. 2C). QVD also partially inhibited RG7112-induced apoptosis in both MLL-5 and MLL-14 xenograft cells after 24 hours treatment (Fig. 2D). These results show that RG7112 induces caspase-dependent apoptosis in both MLL-ALL cell lines and xenograft cells, in contrast with studies with cancer cells of different histotypes (32).

RG7112 activates the p53 pathway in vivo and induces significant regressions in infant MLL-ALL xenografts

The in vivo efficacy of RG7112 was next tested against the infant MLL-ALL xenograft panel described above. Once daily administration of RG7112 for 14 days induced significant and prolonged regressions of seven of seven xenografts (Fig. 3A, Table 1; Supplementary Fig. S5), including the previously reported results for MLL-7 (31). Using stringent objective response criteria modeled after the clinical setting, six xenografts achieved complete responses (CR), and one (MLL-7) had previously sustained a maintained complete response (MCR; ref. 31). RG7112 also
Figure 1.
Characterization of an MLL-ALL xenograft panel by gene-expression profiling. A, unsupervised hierarchical clustering of MLL-ALL (n = 9, red), BCP-ALL (n = 17, yellow), and T-ALL (blue, n = 9) xenografts. B, dendrogram representing hierarchical clustering of the MLL-ALL xenograft panel: t(11;19), green; t(4;11), yellow; t(1;11), purple; t(10;11), blue; t(11;17), red. C, the top 100 genes most highly correlated with either the MLL-ALL or BCP-ALL subtype. Each column represents an individual leukemia xenograft sample, and each row represents a unique gene. Expression levels are normalized for each gene, where the mean is 0. Expression levels greater than the mean are shown in red and less than the mean are in blue. D, heatmap separating the MLL-rearranged from BCP-ALL xenografts based on the MLL-rearranged gene-expression signature published by Armstrong et al. (4). Red arrows indicate genes that were differentially expressed (FDR ≤ 0.05) between MLL-ALL and BCP-ALL xenografts (see Supplementary Table S3 for statistical analysis). Color scaling is as indicated in C.
significantly prolonged mouse EFS compared with vehicle controls in all evaluable xenografts, with LGDs ranging from 17.1 to 44.7 days (Table 1). The 14-day RG7112 treatment was well tolerated and caused no marked effects on mouse weight or hematologic parameters (Supplementary Fig. S6). A complete summary of results is provided in Supplementary Table S4, including total numbers of mice, numbers of mice that died (or were otherwise excluded), numbers of mice with events and median times to events, as well as numbers in each of the ORM categories and “treated over control” (T/C) values. Of note, only 2 of 51 (<4%) of RG7112 mice experienced toxicity-related events, with 45 of 51 (88%) of mice analyzed for leukemia-related events.

To investigate mechanisms associated with the pronounced in vivo efficacy of RG7112 against infant MLL-ALL xenografts, mice highly engrafted with MLL-5 or MLL-14 received a single dose of RG7112 or vehicle control and spleen-derived cells (>95% huCD45+) were harvested 6 to 48 hours later. Immunoblot analysis showed a marked increase in p53 and its transcriptional targets MDM2 and p21WAF1 proteins within 6 hours of treatment (see Fig. 3B for immunoblots representative of a single mouse from each group of 3 mice, and Supplementary Fig. S7 for replicate immunoblots of all MLL-14 engrafted mice). The highest levels of p53 protein were observed at 6 hours posttreatment, but p53 remained elevated until at least 24 hours posttreatment. This activation of p53 was followed by induction of PUMA, and downregulation of survivin (Fig. 3B). These results indicate that RG7112 effectively induces cell-cycle arrest and proapoptotic protein expression patterns in vivo in infant MLL-ALL xenografts.

To further understand the underlying basis for the impressive in vivo efficacy of RG7112 against infant MLL-ALL xenografts, we compared the relative expression of the TP53 and MDM2 genes with respect to in vivo RG7112 efficacy between infant MLL-ALL xenografts in this study, and BCP-ALL xenografts in our previous report (31). Infant MLL-ALL xenografts expressed significantly higher levels of TP53, and significantly lower MDM2 levels, than BCP-ALL xenografts (Supplementary Fig. S8A–S8C). Basal expression of MDM2 ($R^2 = 0.52$, $P < 0.005$, Supplementary Fig. S8D), but not TP53 ($R^2 = 0.026$, $P = 0.58$, Supplementary Fig. S8E) significantly correlated with in vivo sensitivity of ALL xenografts to RG7112.
RG7112, with the paradoxical finding that xenografts expressing lower levels of MDM2 exhibited a longer progression delay. The infant MLL-ALL xenografts had a significantly better response to RG7112 in vivo compared with BCP-ALL xenografts, as assessed by both treated–control EFS and treated–control EFS values (Supplementary Fig. S8F and S8G).

**Synergistic in vitro and in vivo effects of RG7112 with established drugs against MLL-translocated leukemia cells**

Approximately 90% of infants with MLL-ALL initially enter complete remission when treated with combination chemotherapy, although a 5-year EFS is, at best, 50% for these patients (6, 9, 10). Therefore, we next assessed whether RG7112 exhibited the potential to augment the antileukemic effects of the established drugs VXL. Fixed-ratio combination cytotoxicity assays were carried out using ex vivo cultured MLL-ALL xenograft cells and the RS4;11 cell line (Fig. 4). RG7112 exhibited synergistic cytotoxicity with vincristine in 4 of 4 models, with dexamethasone in 2 of 4, and L-asparaginase in 3 of 4 (Fig. 4; Supplementary Table S5).

Because RG7112 exhibited broad in vitro synergistic effects with VXL across four MLL-translocated leukemia models, we next assessed whether these results could be replicated in the in vivo setting. A 4-week VXL induction-type regimen that was recently optimized in our laboratory (38) induced significant progression delays in all MLL-ALL xenografts (Supplementary Table S6). Because RG7112 administered daily for 14 days also exerted impressive single-agent efficacy against all MLL-ALL xenografts...
tested (Fig. 3, Table 1; Supplementary Fig. S5 and Supplementary Table S4), we devised an abbreviated 5-day treatment schedule to achieve measurable responses and indications of therapeutic synergy (see Materials and Methods).

VXL and RG7112 significantly delayed the progression of MLL-5 and MLL-14 compared with vehicle controls (Fig. 5; Supplementary Table S7). RG7112 achieved progressive disease 2 (PD2) in MLL-5 (P < 0.0001) and a CR in MLL-14 (P = 0.001), with LGDs of 11.1 and 17.3 days, respectively. VXL also achieved a PD2 in MLL-5 and CR in MLL-14, with LGDs of 8.0 and 23.5 days, respectively. The combination of RG7112 and VXL significantly extended disease remission to 19.0 days for MLL-5 and increased the ORM from PD2 for each of RG7112 or VXL to a CR for the combination, whereas the LGD for MLL-14 increased to 60.1 days with an ORM of MCR (compared with CRs for RG7112 and VXL alone). Using strict criteria defined in Materials and Methods RG7112 plus VXL caused therapeutic enhancement in MLL-14 (P < 0.01 for the combination vs. both the RG7112 and VXL treatment arms) and a trend for therapeutic enhancement for MLL-5 (P = 0.04 for the combination vs. VXL). These data suggest that combining RG7112 with current remission-induction chemotherapy could prove useful in the management of infant MLL-ALL.

**Discussion**

The NOD/SCID mouse model has previously been shown to be a useful tool for the engraftment and serial passage of primary childhood ALL patient biopsies (33, 34). In addition, it has been demonstrated that in vivo responses of childhood ALL xenografts

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### Table 1. In vivo activity of RG7112 against MLL-ALL xenografts

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<th>Xenograft</th>
<th>Median EFS (days)</th>
<th>LGD (days)</th>
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**NOTE:** Values in bold signify statistically significant drug effects (P < 0.05).

*Data for this xenograft previously published (31).
to commonly used chemotherapeutic agents, dexamethasone, and vincristine, significantly correlated with the clinical outcome of the patients from whom the xenografts were originally derived (34). Here, we report on the establishment and characterization of a new panel of xenografts derived from infant ALL harboring MLL translocations. Seven of nine samples successfully engrafted and were further expanded to quaternary passage, demonstrating the robustness of this xenograft model. Gene-expression profiling revealed that MLL-ALL xenografts cluster separately from BCP-ALL and T-ALL xenografts, and that there was a subset of 2,654 genes that were differentially expressed between the MLL-ALL and BCP-ALL xenografts. These results demonstrate that the unique signature distinguishing MLL-translocated leukemias from BCP-ALL and T-ALL was maintained during serial passage through NOD/SCID mice; an important requirement for useful preclinical models to study novel therapies specifically targeted against this disease.

Not only are p53 mutations uncommon in infant MLL-ALL, but some of the most frequently detected MLL fusion proteins are reported to inhibit p53 transcriptional activity, which may contribute to MLL-induced leukemogenesis and drug resistance (47). Therefore, drugs that release p53 from inhibitory mechanisms could be of therapeutic benefit against this high-risk ALL subtype. We previously examined the in vivo efficacy of RG7112 as a single agent against eight ALL xenografts (five BCP-ALL, two T-ALL, and one infant MLL-ALL; ref. 31). RG7112 demonstrated strong antileukemic activity against all ALL subtypes; however, the most impressive response was observed in the single infant MLL-ALL xenograft (MLL-7) that achieved an LGD of >44 days and an MCR. Here, we report that RG7112 induces significant regressions against a large panel of MLL-ALL xenografts with diverse MLL translocation partners in vivo. RG7112 efficiently stabilized and activated p53 in MLL-ALL xenografts leading to induction of p53-dependent cell-cycle arrest and apoptosis in vivo. In addition to the
induction of p53-dependent proapoptotic targets such as PUMA, we also observed downregulation of the antiapoptotic protein survivin after RG7112 treatment. This is consistent with a previous study reporting that the survivin gene contains a p53 response element, which is activated upon cellular stress resulting in reduced survivin expression (48). The mechanism of death induced by RG7112 in an MLL-ALL cell line and xenografts appeared highly dependent on apoptosis, in contrast with studies with cancer cells of different histotypes (32), which may reflect the greater propensity of lymphoid cells to undergo apoptosis compared with other cell lineages. Taken together, our results suggest that RG7112-mediated cell death in infant MLL-ALL is due to activation of the p53 protein and subsequent p53-mediated transcriptional activity, both through induction of proapoptotic factors and repression of antiapoptotic proteins such as survivin.

Infants diagnosed with MLL-rearranged ALL usually respond to initial induction therapy, but the majority achieves only a shallow remission with detectable molecular disease and these patients frequently relapse on therapy (49). In this study, we showed that remission with detectable molecular disease and these patients initial induction therapy, but the majority achieves only a shallow

totic factors and repression of antiapoptotic proteins such as
ated transcriptional activity, both through induction of proapop-
totic proteins such as survivin.

Disclosure of Potential Conflicts of Interest

P.J. Houghton reports receiving other research grants from Hoffman La Roche. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: J Richmond, H Carol, R Marschalek, P.J. Houghton, M.A. Smith, R.B. Lock
Development of methodology: J Richmond, L High, U.R. Kees, R.B. Lock
Acquisition of data (provided materials, acquired and managed patients, provided facilities, etc.): J Richmond, H Carol, K. Evans, A. Mendomo, A. Robbins, C. Meyer, N.C. Venn, R. Marschalek, R. Sutton, R.B. Lock
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J Richmond, H Carol, K. Evans, L. High, A. Mendomo, R. Sutton, R.T. Kurmasheva, U.R. Kees, P.J. Houghton
Writing, review, and/or revision of the manuscript: J Richmond, H Carol, R. Marschalek, M. Henderson, R. Sutton, U.R. Kees, M.A. Smith, R.B. Lock
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J Richmond, K. Evans, A. Robbins, R.T. Kurmasheva, P.J. Houghton, R.B. Lock
Study supervision: M. Henderson, P.J. Houghton, R.B. Lock

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Jennifer Richmond, Hernan Carol, Kathryn Evans, et al.

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