A high-throughput screen indicates gemcitabine and JAK inhibitors may be useful for treating pediatric AML

Christina D. Drenberg1,2, Anang Shelat3, Jinjun Dang4, Anitria Cotton4, Shelley J. Orwick2, Mengyu Li1,2, Jae Yoon Jeon1,2, Qiang Fu1,2, Daelynn R. Buelow1,2, Marissa Pioso1,2, Shuiying Hu1,2, Hiroto Inaba4, Raul C. Ribeiro4, Jeffrey E. Rubnitz4, Tanja A. Gruber4, R. Kiplin Guy5 & Sharyn D. Baker1,2

Improvement in survival has been achieved for children and adolescents with AML but is largely attributed to enhanced supportive care as opposed to the development of better treatment regimens. High risk subtypes continue to have poor outcomes with event free survival rates <40% despite the use of high intensity chemotherapy in combination with hematopoietic stem cell transplant. Here we combine high-throughput screening, intracellular accumulation assays, and in vivo efficacy studies to identify therapeutic strategies for pediatric AML. We report therapeutics not currently used to treat AML, gemcitabine and cabazitaxel, have broad anti-leukemic activity across subtypes and are more effective relative to the AML standard of care, cytarabine, both in vitro and in vivo. JAK inhibitors are selective for acute megakaryoblastic leukemia and significantly prolong survival in multiple preclinical models. Our approach provides advances in the development of treatment strategies for pediatric AML.
Dramatic improvements in survival have been achieved for children and adolescents with acute myeloid leukemia (AML), with 5-year survival rates increasing between 1975 and 2010 from <20% to >70%\(^1\). These improvements are attributable to the intensification of chemotherapy, selective use of hematopoietic stem cell transplantation, improvements in supportive care, refinements in risk classification, and use of minimal residual disease to monitor response to therapy\(^2\). However, in the past decade outcome has plateaued and remain unacceptably low for many subtypes. Provided that significant improvements in long-term outcome are not expected with conventional therapy alone, therapeutic strategies that can be quickly advanced to a clinical setting are urgently needed for the treatment of pediatric AML.

Drug discovery and development is a long process that requires an enormous financial investment and multiple clinical trials; a process that has an increased number of challenges and barriers in orphan diseases such as pediatric AML. A more rational, evidence-based approach to identify and prioritize therapeutics advancing to clinical trials is needed. Here, we report the results of a large-scale screen of human cancer cell lines representing two high-risk subtypes of pediatric AML including MLL rearranged (MLLr) with or without a co-occurring FLT3-internal tandem duplication (FLT3-ITD) mutation and non-Down syndrome acute megakaryoblastic leukemia (AMKL)\(^2,3\). Both FLT3-ITD and MLLr occur in adult AML patients and are associated with poor prognosis whereas, AMKL is extremely rare occurring in only 1% of the adult patient population\(^5,7\). This provides a strong rationale for identifying treatment strategies that may be unique to pediatric AML and those that may have broader implications. The Broad and Sanger Institutes independently reported the findings are consistent with our previous report with panobinostat\(^13\) and support the ongoing clinical evaluation (NCT02676323) of this drug for pediatric AML. Similarly, the proteasome inhibitors, carfilzomib and bortezomib, demonstrated potent activity and have been extensively investigated in the clinic\(^14\). Criterion for the advancement of compounds are described in the Methods section and may permit rapid translation to a clinical trial in pediatric AML; this is essential as we currently have access to few if any investigational agents.

### Results

**Drug screen using AML cell lines.** We validated a panel of 8 AML cell lines derived from children and young adults for screening (Fig. 1a, Supplementary Fig. 1, Supplementary Table 1). A primary HTS was performed using a library of 7389 compounds (6568 unique) at a single concentration. Percent inhibition of cell proliferation was determined relative to the positive control (Supplemental Data 1). Assay diagnostics were acceptable and the scatter-plot of controls and test compound activity demonstrated adequate separation between signal and noise for each cell line (Supplementary Fig. 2). The range of total hits (activity >50% inhibition) in the primary screen was 334–624 and the number of selective hits (activity >80% in one cell line and <20% in all others) was negligible (range 0–6; mean 2.6) (Supplementary Table 2). A secondary HTS performed in a dose-response manner, included FDA approved compounds with inhibition >50% in more than one cell line in the primary screen, analogs of these hits, and other compounds of interest (e.g., NAMPT inhibitors) not included in the primary screen; clinical phase of testing was also taken into consideration. The average effective concentration of each compound is reported in Fig. 1b and Supplementary Data 2. Of the 458 compounds, we identified 17 with potency <1 µM in all cell lines; collectively, these included histone deacetylase inhibitors (S/7), proteasome inhibitors (4/17), PI3K inhibitor (1/17), inhibitors of anti-apoptotic proteins (2/17), and FDA-approved cytotoxic agents (S/7) two of which (clofarabine, mitoxantrone) are currently used in clinical regimens for the treatment of AML (Table 1). We validated compounds from these drug classes and others targeting pathways known to be upregulated or mutated in MLLr or AMKL (e.g., trametinib, RAS pathway; alisertib, aurora kinase; RG7112, MDM2 inhibitor)\(^10,12\) using cell lines and primary patient samples (Fig. 2, Supplementary Fig. 3, Supplementary Table 3). Primary patient samples were co-cultured with mesenchymal stromal cells, which secrete multiple cytokines that mimic the bone marrow microenvironment; this system gives support to primary samples while challenging the drug treatment. Cell viability and cell density were monitored throughout the assay (Supplementary Fig. 4); these data demonstrate all primary samples experience a dramatic decrease in cell number at 24 h; though cell numbers are relatively maintained over the course of the assay only one sample doubles from 24 to 96 h. This is an important observation especially in regard to drugs that specifically target S phase cells and may contribute to the modest activity of nucleoside analogues like cytarabine and gemcitabine in this assay. We observed the HDAC inhibitors panobinostat and romidepsin to have potent activity across subtypes; these findings are consistent with our previous report with panobinostat\(^13\) and support the ongoing clinical evaluation (NCT02676323) of this drug for pediatric AML. Similarly, the proteasome inhibitors, carfilzomib and bortezomib, demonstrated potent activity and have been extensively investigated in the clinic\(^14\). Criterion for the advancement of compounds are described in the Methods section and may permit rapid translation to a clinical trial in pediatric AML; this is essential as we currently have access to few if any investigational agents.

Gemcitabine demonstrates potent in vitro activity. Given that nucleoside analogs are integral to all modern AML therapy and since gemcitabine, a nucleoside analog that is currently used to treat advanced solid tumors in children\(^15,16\) demonstrated very potent activity across subtypes in the secondary HTS (<65 nM); and taken these results were validated in a low-throughput manner (Fig. 3a) and had comparable activity to cytarabine in primary patient samples (Fig. 2, Supplementary Table 3), we selected this compound for further evaluation. A panel of AML cell lines were treated with increasing concentrations of gemcitabine and clinically used nucleoside analogs for comparison. Both cytarabine and fludarabine demonstrated variable activity; whereas both clofarabine and gemcitabine had more narrow half maximal inhibitory concentration (IC\(_{50}\)) ranges (Fig. 3a). Overall, gemcitabine was the most potent and had activity in cell lines that were insensitive to cytarabine (THP-1) and fludarabine (CHRF288-11, MV4-11).
Collectively, nucleoside analogs share a similar mechanism of action whereby they enter cells exclusively by transporter-mediated processes\(^1\). Upon intracellular uptake, multiple rounds of phosphorylation must occur before insertion into DNA\(^1\). Reduced uptake into leukemia cells has been proposed as a process underlying most instances of clinical resistance to cytarabine, though the responsible mechanism remains poorly understood\(^2\). To investigate whether differences in cellular uptake were contributing to the enhanced anti-leukemic activity of gemcitabine, we performed intracellular uptake and accumulation experiments. In a comparative analysis after 5 min exposure to cytarabine or gemcitabine, we detected a significantly greater...

Fig. 1 High-throughput screening of pediatric AML. a Illustration of the scheme used for AML cells in the screening platform. Optimal plating density was determined per cell line in a 384-well plate; a primary screen was conducted at a single concentration and a secondary screen was performed in a dose-response manner in triplicate. For screens, cells were plated and after 24 h compounds were pin tool transferred using an automation station. Cell viability was measured at 72 h using Cell Titer Glo. Select compounds were validated using cell lines, primary patient samples, and/or in vivo murine models. b Heatmap of the average effective concentration (EC\(_{50}\)) from secondary screen. Cell lines are ordered based on a cluster analysis. Black, MLL rearranged (MLL\(_r\)); blue, FLT3-internal tandem duplication positive with MLL\(_r\); green, PICALM/MLLT10 fusion positive; red, acute megakaryoblastic leukemia. Color bar on top of heatmap indicates compound classes: red, anti-infective and anti-psychotic; orange-red, anti-metabolite; orange, apoptosis; yellow, DNA damage; lime, complex; green, folate, epigenetic, retinoic acid receptor; teal, Hsp90; light blue, kinase; blue, microtubule, NF-\(\kappa\)B; purple, other; light pink, proteasome; pink, HIF, Nrf2; NE, not evaluated.

Collectively, nucleoside analogs share a similar mechanism of action whereby they enter cells exclusively by transporter-mediated processes\(^1\). Upon intracellular uptake, multiple rounds of phosphorylation must occur before insertion into DNA\(^1\). Reduced uptake into leukemia cells has been proposed as a process underlying most instances of clinical resistance to cytarabine, though the responsible mechanism remains poorly understood\(^2\). To investigate whether differences in cellular uptake were contributing to the enhanced anti-leukemic activity of gemcitabine, we performed intracellular uptake and accumulation experiments. In a comparative analysis after 5 min exposure to cytarabine or gemcitabine, we detected a significantly greater...
amount (1.2–3.6-fold) of gemcitabine in all cell lines evaluated (Fig. 3b). This observation continued over a 2-h time course and was associated with significantly increased gemcitabine accumulation (5–19.8-fold compared to cytarabine) in the nuclear compartment (Fig. 3c, d). While the observed nuclear uptake suggests that the active metabolite gemcitabine triphosphate (dFdCTP) was accumulating to a much greater extent relative to cytarabine triphosphate (Ara-CTP); these studies detect total radioactivity, which is comprised of both active and inactive metabolites. To determine if we were observing greater accumulation of dFdCTP, we evaluated the accumulation of gemcitabine monophosphate (dFdCMP), diphosphate (dFdCDP), and dFdCTP compared to the metabolic counterparts of cytarabine (Ara-CMP, Ara-CDP, Ara-CTP). After 2 h exposure, significantly greater accumulation of dFdCTP compared to Ara-CTP (7.3–61.2-fold) was observed; dFdCTP accounted for 58–81% of the total intracellular accumulation in all cell lines evaluated whereas Ara-CTP accounted for 52–65% (Fig. 3e, Supplementary Fig. 5a–c). Additionally, we evaluated the accumulation using primary murine blasts isolated from the bone marrow and spleen of treatment-naive MllPTD/wt:Flt3ITD/ITD double knock-in primary transplants. Similarly, we observed higher accumulation of total gemcitabine versus cytarabine (6.0–6.7-fold) and dFdCTP versus Ara-CTP (2.4–5.3-fold) in blasts from bone marrow or spleen (Supplementary Fig. 5d).

To determine if dose escalation could result in equivalent intracellular exposure, we performed uptake assays at higher concentrations of cytarabine. After 5 min exposure to cytarabine (10 or 100 µM) we detected a significantly greater amount (1.6–3.7-fold and 7–12-fold, respectively) compared to 1 µM cytarabine (Fig. 3f). Furthermore, we found a 10-fold higher concentration of cytarabine produced nearly equivalent intracellular exposure to 1 µM gemcitabine. Since transporters are biophysically complex and the interpretation of

Table 1 Compounds with EC50 <1 µM in all cell lines evaluated in secondary HTS

| Compound       | MOLM-13 | MV4-11 | CHRF288-11 | CMK | CMS | M07e | ML2 | U937 |
|----------------|---------|--------|------------|-----|-----|------|-----|------|
| Clofarabine    | 0.05    | 0.15   | 0.37       | 0.005 | 0.02 | 0.01 | 0.13 | 0.09 |
| Gemcitabine    | 0.003   | 0.002  | 0.003      | 0.01 | 0.03 | 0.06 | 0.002 | 0.01 |
| Dactinomycin   | 0.03    | 0.04   | 0.001      | 0.01 | 0.04 | 0.001 | 0.001 | 0.001 |
| Mitoxantrone   | 0.01    | 0.001  | 0.02       | 0.04 | 0.08 | 0.03 | 0.24 | 0.03 |
| Trichostatin   | 0.08    | 0.04   | 0.04       | 0.05 | 0.04 | 0.09 | 0.11 | 0.04 |
| Quisinostat    | 0.01    | 0.01   | 0.01       | 0.02 | 0.001 | 0.004 | 0.004 | 0.01 |
| CUDC-907       | 0.0004  | 0.0002 | 0.001      | 0.002 | 0.001 | 0.001 | 0.002 | 0.004 |
| Camptothecin   | 0.18    | 0.13   | 0.01       | 0.03 | 0.03 | 0.006 | 0.01 | 0.01 |
| Romidepsin     | 0.001   | 0.001  | 0.01       | 0.01 | 0.001 | 0.002 | 0.004 | 0.003 |
| NVP-BG7286     | 0.11    | 0.05   | 0.05       | 0.06 | 0.01 | 0.25 | 0.23 | 0.08 |
| Cabazitaxel    | 0.001   | 0.0003 | 0.001      | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 |
| Ouabain        | 0.002   | 0.002  | 0.002      | 0.01 | 0.001 | 0.001 | 0.001 | 0.02 |
| Bortezomib     | 0.01    | 0.01   | 0.03       | 0.01 | 0.001 | 0.01 | 0.001 | 0.01 |
| Oprozomib      | 0.07    | 0.05   | 0.17       | 0.23 | 0.11 | 0.08 | 0.04 | 0.13 |
| Carfilzomib    | 0.01    | 0.001  | 0.02       | 0.01 | 0.001 | 0.001 | 0.003 | 0.01 |

Mechanism of action indication as follows: a—anti-metabolite; c—complex; d—DNA; e—epigenetic; k—kinase; m—mitotic; o—other; p—proteasome.
porter dynamics can be difficult to quantify with increasing concentrations (Fig. 3g).

Overall transport efficiency of gemcitabine was significantly higher compared to cytarabine and this could not be overcome with increasing concentrations (Fig. 3g).

**Fig. 3** Activity of gemcitabine in AML cell lines. a AML cells were treated with increasing concentrations of vehicle or nucleoside analog (gemcitabine, Gem; cytarabine, Ara-C; clofarabine, Clo; and fludarabine, Flud) for 72 h; cell viability was measured using Cell Titer Glo. Data are reported as percent control and represented as mean ± standard deviation (SD) of 3 independent experiments performed in triplicate (N = 18 per concentration). The half maximal inhibitory concentration (IC50, dotted line) was evaluated by nonlinear regression analysis using GraphPad Prism. Uptake/accumulation assays used a mixture of unlabeled and [3H]-Ara-C (blue circles) or Gem (magenta circles). b Intracellular uptake (5 min, 1 µM) of Ara-C and Gem. Data are mean ± SD from 3 independent experiments performed in triplicate (N = 9). c Time-dependent accumulation (range, 5–120 min; 1 µM) of Ara-C (dashed line) or Gem (solid line) in MOLM-13 (magenta), ML-2 (blue), and CHRF288-11 (black). Data are mean ± SD from 3 independent experiments performed in triplicate (N = 9). d Nuclear accumulation (2 h, 1 µM) of Ara-C and Gem. Data are mean ± SD from 3 independent experiments performed in triplicate (N = 9). e Intracellular Ara-C, Gem, and phosphorylated metabolites (Ara-CMP, Ara-C monophosphate; Ara-CDP, Ara-C diphosphate; Ara-CTP, Ara-C triphosphate; dFdCMP, Gem monophosphate; dFdCDP, Gem diphosphate; dFdCTP, Gem triphosphate) was determined by HPLC coupled with liquid scintillation counting (2 h, 1 µM). Data are mean ± standard error (SE) from 3 independent experiments (N = 3). f Uptake (5 min) of 10 µM (solid blue circles) or 100 µM (open blue circles) Ara-C. Data are mean ± SD from 3 independent experiments performed in triplicate (N = 9). g Transport efficiency was determined for uptake (5 min) assays for Ara-C (blue; circle, 1 µM; square, 10 µM; triangle, 100 µM) and Gem (magenta circle, 1 µM). Data are mean ± SD from 3 independent experiments (N = 9). h Confirmation of target knockdown in MOLM-13 cells at 48 h after transient transfection with siRNA-Control, -OCTN1, or -ENT1; data are mean ± SE from 4 independent experiments performed in triplicate (N = 12). i Cytotoxicity was assessed following 16 h exposure to 100 nM Ara-C or 10 nM Gem by induction of S-phase of the cell cycle in transfected cells. Data are mean ± SE from 3 independent experiments (N = 3). Student’s t-test. *P < 0.05; **P < 0.01; ***P < 0.0001

Michaelis–Menten parameters with regard to underlying transporter dynamics can be difficult to quantify with increasing concentrations (Fig. 3g).

Given that previous investigations demonstrated that low cytarabine uptake in AML cells predicts poor response to therapy and taken our data showing uptake and transport efficiency as a major contributor to the enhanced sensitivity of AML cells to gemcitabine we performed knockdown experiments targeting two key transporters involved in nucleoside uptake OCTN1 and ENT1. Using siRNA we were able to achieve a...
54% and 41% reduction in expression of OCTN1 and ENT1, respectively (Fig. 3h). At 24 h post-transfection, cells were treated with PBS, gemcitabine, or cytobane and alterations in their cytotoxicity profile were assessed using perturbations in cell cycle as the read-out. Inhibition of both transporters resulted in a decrease of cytobane- and gemcitabine-mediated accumulation of cells in S-phase; the effect was greater with gemcitabine in ENT1 deficient cells and comparable in both OCTN1 and ENT1 deficient cells with cytobane (Fig. 3i).

**Taxanes demonstrate potent in vitro activity.** Another class of cytotoxic agents demonstrating potent activity across subtypes were microtubule poisons, specifically taxanes. Cabazitaxel demonstrated potent single agent activity in the secondary HTS and primary patient samples (Figs. 1b and 2, Table 1); likewise, docetaxel demonstrated potent activity in all but one cell line (Supplementary Data 2). These results are consistent with a recent report showing that primary AML cells were sensitive to docetaxel when drug sensitivity and resistance testing was performed in the presence of HS-5 human bone marrow stromal cell conditioned media. We validated the results from the HTS and found cabazitaxel, docetaxel, and paclitaxel have potent activity in a panel of AML cell lines (Fig. 4a); cabazitaxel was the most potent so we selected this compound for further evaluation.

Microtubule poisons like taxanes interfere with the faithful segregation of chromosomes by binding tubulin and disrupting the mitotic spindle causing a G2/M cell cycle arrest, leading to mitotic catastrophe, and ultimately resulting in cell death. Therefore, we assessed the mechanism by which cabazitaxel triggers cell death in AML cells by analyzing cell cycle perturbations using a biparametric approach of markers associated with mitosis. A time-course analysis showed that cell cycle blockage was detected prior to cell death: G2/M arrest was detectable at 4 h and more prominent at 8 and 12 h, whereas cell death was detectable at 12 h and more pronounced at 24 h (Fig. 4b, Supplementary Fig. 6). The G2/M arrest was accompanied by a significant induction of cyclin B and phosphorylation of histone H3 (Fig. 4c). These results suggest that cabazitaxel induces a mitotic arrest in AML cells, which leads to cell death.

**Establishment of AMKL murine models.** Development and implementation of preclinical in vivo models that faithfully recapitulate human disease are imperative to enhance the predictive power of potential therapeutics. In an effort to establish murine models for AMKL, we labeled CHRF288-11 cells with YFP/luciferase (CHRF288-11-Luc+) to permit monitoring engraftment through bioluminescence imaging; we found CHRF288-11-Luc+ cells could engraft in both female and male mice (Supplementary Fig. 7). We have previously identified a pediatric-specific rearrangement yielding a CBFA2T3/GLIS2 fusion which is associated with aberrant JAK/STAT signaling and often co-occurs with mutations in JAK kinase family members, STAT genes, or the thrombopoietin receptor MPL; patients with this lesion show the strongest negative association with survival and highest cumulative incidence of relapse or primary resistance. While expression of this gene fusion results in increased self-renewal, transplantation of fusion gene-modified bone marrow cells fails to induce leukemia suggesting there is an essential requirement for cooperating mutation(s) in cases expressing the gene fusion. We evaluated the transforming potential of dual expression of CBFA2T3/GLIS2 with a clinically relevant JAK2V617F cooperating mutation (CG/V617); concurrent expression uniformly induced a rapid and fatal leukemia characteristic of AMKL and transplantable into subsequent recipients (Supplementary Fig. 8). We then incorporated luciferase into CG/V617 AMKL blasts isolated from secondary transplants (CG/V617-Luc+). The bioluminescence signal could be detected in the hind limbs 15 days earlier than CG/V617 cells in the peripheral blood and CG/V617-Luc+ transplants exhibited a highly penetrant phenotypically similar AMKL to the CG/V617 transplants (Supplementary Fig. 8e). We also established a patient-derived xenograft (PDX) using a sample from a pediatric patient with AMKL that was previously identified to carry the CBFA2T3-GLIS2 fusion plus copy number alterations and amplification on chromosome 21, a major cooperating event that includes genes in the Down Syndrome critical region. These models replicate many features of human AMKL and provide a robust tool set for preclinical evaluation of therapeutic strategies.

**Gemcitabine and cabazitaxel prolong in vivo survival.** Next, we sought to compare the in vivo efficacy of gemcitabine and cabazitaxel to the standard of care, cytarabine. Due to limitations with tolerability, we have previously treated our AML xenograft models with low-dose cytarabine. This regimen did not provide any survival advantage compared to vehicle treated mice (median survival 26 versus 26 days) in the CHRF288-11-Luc+ model (Supplementary Fig 9). Tolerability studies of gemcitabine at the same dose and schedule was not tolerated. Therefore, we performed tolerability using multiple doses on an intermittent every 3 or 4-day schedule; all regimens of gemcitabine were well tolerated. Similarly, we performed tolerability of cabazitaxel using multiple doses on an intermittent every 3 or 4-day schedule; the only tolerable dose was 5 mg/kg.

For efficacy studies performed using immunocompromised mice, we found cytarabine did not provide any survival advantage compared to vehicle treated mice in cell line xenografts; whereas in the AMKL PDX cytarabine significantly prolonged survival (log-rank test, P = 0.0023) (Fig. 5, Supplementary Figs. 10–11). In the AMKL xenograft models, gemcitabine provided the greatest survival advantage and significantly prolonged survival versus cytarabine after one (log-rank test, P = 0.0023) or two treatment cycles (log-rank test; CHRF288-11-Luc+, P = 0.0019; AMKL PDX, P = 0.0026). Treatment with gemcitabine significantly inhibited tumor burden compared to cytarabine as indicated by a reduced bioluminescence signal and decreased infiltration in the peripheral blood. While cabazitaxel significantly prolonged survival versus cytarabine after one (log-rank test, P = 0.0023) or two cycles (log-rank test, P = 0.0023) in the CHRF288-11-Luc+ xenograft, we did not observe a significant difference in the AMKL PDX (Fig. 5, Supplementary Fig. 10). Surprisingly, we found the additional treatment cycle of cabazitaxel in the CHRF288-11-Luc+ model did not further prolong survival compared to one cycle. Although gemcitabine significantly prolonged survival versus cytarabine in the ML-2 xenograft model (log-rank test, P = 0.0027); cabazitaxel provided the greatest survival advantage (log-rank test, P = 0.0027) and significantly inhibited tumor burden compared to cytarabine (Fig. 5c, Supplementary Fig. 11).

Next, we sought to evaluate efficacy in two syngeneic murine models using a maximum tolerated dose (MTD) of cytarabine that better reflects clinical regimens. Tolerability of gemcitabine was performed using multiple doses on an intermittent or daily schedule (Supplementary Fig. 12) to establish the MTD. For efficacy studies in CG/V617-Luc+ quaternary transplants, mice were treated with gemcitabine (MTD daily or intermittent) or cytarabine (daily). We found both dosing regimens of cytarabine provided a survival advantage compared to vehicle treated mice (log-rank test, P = 0.0091) (Fig. 5d). However, gemcitabine provided the greatest survival advantage (log-rank test, daily, P = 0.0091; intermittent, P = 0.004) and significantly prolonged
Fig. 4 Activity of taxanes in AML cell lines. a AML cells were treated with increasing concentrations of vehicle or taxane (cabazitaxel, Cab; docetaxel, Doce; paclitaxel, Pac) for 72 h, and cell viability was measured using Cell Titer Glo. Data are reported as percent control and represented as mean ± standard deviation (SD) of 3 independent experiments (N = 18 per concentration). The half maximal inhibitory concentration (IC50) was evaluated by nonlinear regression analysis using GraphPad Prism. CHRF288-11, ML-2, and MOLM-13 cells were treated with 5 or 50 nM Cab or DMSO for 12 h. b Cell cycle distribution was determined by DAPI staining and c induction of mitosis was determined by biparametric flow cytometry using p-histone H3 and cyclin B1. Data are mean ± standard deviation from 3 independent experiments (N = 3); Student’s t test. *P < 0.01; **P < 0.001; ***P < 0.0001
survival versus cytarabine (log-rank test, \( P = 0.0027 \)). Treatment with gemcitabine significantly inhibited tumor burden compared to cytarabine as indicated by decreased infiltration in the peripheral blood (Fig. 5d).

Lastly, we evaluated efficacy using a Mll-PTD/wt:Flt3ITD/ITD double knock-in murine model\(^3^2,3^3\). Primary transplants were treated with intermittent dosing of gemcitabine, cytarabine, or cabazitaxel; whereas, secondary transplants were treated with MTD of gemcitabine (daily or intermittent) or cytarabine (daily). In primary transplants, cytarabine did not significantly prolong survival compared with vehicle; whereas transplants treated with cabazitaxel (log-rank test, \( P = 0.011 \)) achieved a significant
ITD-double knock-in model. Kaplan–Meier analysis (top panels) of animal survival in a CHRF288-11-luciferase/YFP+ xenograft, b AMKL patient-derived xenograft, c ML-2-luciferase/YFP+ xenograft, d CBFA2T3-GLIS2/JAK2V617F-induced AMKL model, and e, f MLL-PTD/wt-FLT3ITD/ITD-double knock-in model. a-c, e Mice were randomized to receive vehicle (black), 50 mg kg⁻¹ cytarabine (Ara-C, blue), gemcitabine 50 mg kg⁻¹ (Gem, magenta), or cabazitaxel 5 mg kg⁻¹ (Cab, gray) once every 4 days (q4d) for 3 weeks for up to 2 cycles; or d, f mice were randomized to receive vehicle (black), 50 mg kg⁻¹ cytarabine (Ara-C, blue, solid line), 100 mg kg⁻¹ cytarabine (Ara-C, blue, dashed line), or gemcitabine 3 mg kg⁻¹ (Gem, magenta, solid line) once daily for 5 days for up to 3 cycles or 120 mg kg⁻¹ gemcitabine (Gem, magenta, dashed line) every once 3 days (q3d) for 3 weeks for up to 1.5 cycles. All treatments were administered by intraperitoneal injection, black arrows indicate treatment schedule per model. Tumor burden was monitored (bottom panels) by a, c bioluminescent imaging, b detection of human CD45+ cells in peripheral blood, d detection of GFP/mCherry double positive (DP) cells in peripheral blood, or e, f detection of Ly5.2+ cells in peripheral blood. Student’s t test; NS not significant; *P < 0.05; **P < 0.01; ***P < 0.001

Fig. 5 In vivo activity of gemcitabine and cabazitaxel. Kaplan–Meier analysis of animal survival in the CHRF288-11-luciferase/YFP+ xenograft, AMKL patient-derived xenograft, ML-2-luciferase/YFP+ xenograft, CBFA2T3-GLIS2/JAK2V617F-induced AMKL model, and MLL-PTD/wt-FLT3ITD/ITD-double knock-in model. a-c, e Mice were randomized to receive vehicle (black), 50 mg kg⁻¹ cytarabine (Ara-C, blue), gemcitabine 50 mg kg⁻¹ (Gem, magenta), or cabazitaxel 5 mg kg⁻¹ (Cab, gray) once every 4 days (q4d) for 3 weeks for up to 2 cycles; or d, f mice were randomized to receive vehicle (black), 50 mg kg⁻¹ cytarabine (Ara-C, blue, solid line), 100 mg kg⁻¹ cytarabine (Ara-C, blue, dashed line), or gemcitabine 3 mg kg⁻¹ (Gem, magenta, solid line) once daily for 5 days for up to 3 cycles or 120 mg kg⁻¹ gemcitabine (Gem, magenta, dashed line) every once 3 days (q3d) for 3 weeks for up to 1.5 cycles. All treatments were administered by intraperitoneal injection, black arrows indicate treatment schedule per model. Tumor burden was monitored (bottom panels) by a, c bioluminescent imaging, b detection of human CD45+ cells in peripheral blood, d detection of GFP/mCherry double positive (DP) cells in peripheral blood, or e, f detection of Ly5.2+ cells in peripheral blood. Student’s t test; NS not significant; *P < 0.05; **P < 0.01; ***P < 0.001.

Pharmacological assessment of ruxolitinib. Next, we determined the pharmacokinetic properties of ruxolitinib following oral administration in the two mouse strains used for efficacy studies. A single dose of ruxolitinib was administered and total plasma concentrations of ruxolitinib were measured over a 2 h time-course. Concentration-time profiles and a summary of ruxolitinib pharmacokinetic parameters are shown in Fig. 7a, b and Supplementary Table 4. Although we observed strain differences, overall the area under the curve values and half-life approach those observed clinically in children with relapsed cancers.

Ruxolitinib prolongs in vivo survival in multiple models. Finally, we evaluated the in vivo efficacy of ruxolitinib using multiple murine models of AMKL. We found that ruxolitinib significantly prolonged survival compared to vehicle in all three models (log-rank test; CHRF288-11-Luc+, P = 0.003; CG-V617-Luc+ quaternary transplants, P = 0.004; AMKL PDX, P = 0.005) and significantly inhibited tumor burden compared to vehicle as indicated by a reduced bioluminescence and decreased infiltration in the peripheral blood (Fig. 7c–e).
Fig. 6 Activity of JAK inhibitors for AMKL. a AMKL cells were treated with increasing concentrations of vehicle or JAK inhibitor (tofacitinib, ruxolitinib) for 72 h, and cell viability was measured using Cell Titer Glo. Data are reported as percent control and represented as mean ± standard deviation (SD) of 3 independent experiments (N = 18 per concentration). The half maximal inhibitory concentration (IC50, dotted line) was evaluated by nonlinear regression analysis using GraphPad Prism. b Expression of JAK-STAT family members was determined by RNAseq in AMKL cell lines (blue; CHRF288-11, CMK, CMS, CMY, M-MOK, M07e, WSU; N = 7) compared to non-AMKL cell lines (black; ML-2, MOLM-13, MV4-11, U937; N = 4). Data are mean ± standard deviation (SD). c Confirmation of STAT5A expression using a TaqMan expression assay was performed in an expanded panel of AML cell lines (blue circle, AMKL; magenta circle, HEL; black, non-AMKL) and normalized to GAPDH (left). Data are mean ± SD (N = 6). Mean per group ± SD (right). d Correlation analysis of STAT5A expression and ruxolitinib (Rux) IC50 in AMKL cell lines was determined by Pearson correlation and linear regression. e Protein expression of total STAT5A (t-STAT5A) and phospho-STAT5 (p-STAT5) and t-STAT5; GAPDH was used as a loading control. f AMKL cell lines (CHRF288-11, M07e) were exposed to their respective ruxolitinib IC50 (143 and 45 nM) for 1 h then lysed. Western blot analysis was performed on whole cell lysate to evaluate protein expression of phospho-STAT5 (p-STAT5) and t-STAT5; GAPDH was used as a loading control. g CHRF288-11 cells were treated with increasing concentrations of ruxolitinib (magenta) with or without 100 ng mL−1 of BMP2 (black), EPO (blue), or TPO (gray) for 72 h and cell viability was measured using Cell Titer Glo. Data are reported as percent control and represented as mean ± SD of 3 independent experiments (N = 18 per concentration). The IC50 (dotted line) was evaluated by nonlinear regression analysis using GraphPad Prism. h CHRF288-11 cells were treated with 100 ng mL−1 of TPO for up to 1 h and lysed; western blot analysis was performed on whole cell lysate to evaluate protein expression of p-STAT5 and t-STAT5; GAPDH was used as a loading control. Student’s t test; **P < 0.01; ***P < 0.0001

Discussion

Despite many advances in the treatment of pediatric AML, the long-term survival is still unacceptably low and new therapeutic strategies are urgently needed for patients with high-risk subtypes. Using an integrated approach we have identified alternative chemotherapeutic regimens can be superior to standard of care and targeted agents may be useful for select subtypes. Collectively, our data suggest that treatment success could be improved through a repurposing strategy.

Nucleoside analogs have been widely used in the treatment of hematologic malignancies of which cytarabine is the mainstay of therapy in AML41. Cytarabine and gemcitabine are structurally similar nucleoside analogs that require cellular uptake and activation through multiple intracellular phosphorylation steps. While both drugs are activated by the same enzymes, gemcitabine has additional mechanisms of action and a pattern of self-potentiation that is unique among nucleoside analogs42,43. Due to its higher affinity for deoxycytidine kinase gemcitabine undergoes greater activation to mono- and di-phosphorylated metabolites compared to cytarabine and fludarabine43,44. Further, dFdCDP is reported to inhibit ribonucleotide reductase, an enzyme in the nucleotide pathway and critical for management of

![Graph showing the activity of JAK inhibitors for AMKL.](image)
**Table 2 Ruxolitinib and tofacitinib in vitro kinase binding and inhibition**

| Target in binding assaya | $K_d$ Ruxolitinib (nM) | $K_d$ Tofacitinib (nM) | IC$_{50}$ Ruxolitinib (nM) | IC$_{50}$ Tofacitinib (nM) |
|--------------------------|------------------------|------------------------|-----------------------------|-----------------------------|
| JAK1 H1 domain-catalytic  | 11                     | 5.2                    | 0.54                        | 1.9                         |
| JAK1 JH2 domain-pseudokinase | 15,000              | 30,000                 | 0.47                        | 6.2                         |
| JAK2 JH1 domain-catalytic | 0.054                 | 0.59                   | 1.5                         | 2.4                         |
| JAK3 JH1 domain-catalytic | 0.87                  | 0.18                   | 14.7                        | 0.97                        |
| TYK2 JH1 domain-catalytic | 0.25                  | 6.4                    | 0.45                        | 25                          |
| TYK2 JH2 domain-pseudokinase | 2200                | 30,000                 |                             |                             |

aData obtained from KdELECT

---

**Fig. 7** Pharmacokinetics and in vivo activity of ruxolitinib. Pharmacokinetic profile in female (magenta) and male (blue) a NSG and b BoyJ mice treated with a single dose of ruxolitinib (60 mg kg$^{-1}$, oral gavage). Serial blood sampling was performed and ruxolitinib plasma concentrations were determined by LC-MS/MS. Data are mean ± standard error (SE) (N = 5 per gender). Concentration-time data were analyzed by non-compartmental analysis and pharmacokinetic parameters were calculated in WinNonLin. c-e, top Kaplan–Meier analysis of animal survival in c CHRF288-11-luciferase/YFP+ xenograft, d CBF23T3-GLIS2/JAK2V617F-induced AMKL quaternary transplants, and e AMKL patient-derived xenograft. Mice were randomized to receive vehicle (black) or ruxolitinib (blue; 60 mg kg$^{-1}$) twice daily for 5 days for up to 4 weeks; black arrows indicate treatment schedule per model. c-e, bottom Tumor burden was monitored by c, d bioluminescent imaging or e detection of human CD45+ cells in peripheral blood; data are mean ± SE. Student’s t test; *P < 0.01; **P < 0.0001
deoxynucleotide pools in cancer cells.\textsuperscript{45} Incorporation of dFdCTP into DNA results in masked chain termination, where one additional deoxynucleotide is incorporated before termination of DNA synthesis. This specific type of nucleotide linkage masks the gemcitabine nucleotide and prevents recognition by exonuclease making repair difficult.\textsuperscript{44,46} These mechanisms are likely contributing to the significantly greater anti-leukemic activity of gemcitabine that we observed.

Besides inherent mechanisms of action we attribute the enhanced activity of gemcitabine to mechanisms of drug uptake and efflux, processes that contribute to intracellular accumulation. This is supported by our observations demonstrating a significantly greater accumulation of gemcitabine and metabolites. Our observation that the rate of accumulation of dFdCTP was linear up to 2 h is consistent with the results of Gandhi and Plunkett, who found accumulation of gemcitabine to be linear up to 3 h in K562 cells.\textsuperscript{47} Together the rate of drug uptake, efficiency of phosphorylation, and efflux influence the rate of triphosphate formation and cellular retention for both cytarabine and gemcitabine, which ultimately impacts cytotoxicity. We performed uptake assays at increased concentrations of cytarabine and demonstrated that a 10-fold higher uptake was necessary to achieve similar intracellular exposure compared to gemcitabine. These results are consistent with a previous report by Hertel et al.,\textsuperscript{48} which demonstrated a minimum effective concentration of cytarabine is 10-fold higher than gemcitabine in CCRF-CEM cells (T lymphoblastoid cell line) and our own data showing a higher IC\textsubscript{50} for cytarabine compared to gemcitabine. Here, we show the transport efficiency of both drugs and demonstrate that even at escalated concentrations of cytarabine, AML cells have the capacity to transport gemcitabine more efficiently which contributes to the observed differences in intracellular accumulation and cytotoxicity.

While the human ENT1 is thought to be the primary transporter mediating drug influx, we recently identified that entry of cytarabine and several structurally related nucleosides, including gemcitabine, is facilitated by the ergothioneine uptake transporter OCTN1 (SLC22A4; ETT); and low expression of OCTN1 in leukemia cells is a strong predictor of poor survival in multiple cohorts of patients with AML treated with cytarabine-based regimens.\textsuperscript{20} In a parallel study, we reported that cytarabine and Ara-CMP are sensitive to multi-drug resistant protein 4 (MRP4)-mediated efflux, thereby decreasing its cytotoxic response against AML blasts.\textsuperscript{49} In contrast, a similar study found that gemcitabine and its metabolites are not effluxed by MRP4 or MRP5.\textsuperscript{50} It can thus be postulated that differential expression and activity of uptake transporters and their affinities for nucleoside analogs, phosphorylating enzymes that result in active metabolites, and MRPs that mediate drug efflux, play a crucial role in the differential anti-leukemic activity of these compounds. This is consistent with our previous report showing over-expression of OCTN1 in HEK293 cells resulted in increased sensitivity to multiple nucleoside analogs, including cytarabine\textsuperscript{20} and our current findings that inhibition of OCTN1 and ENT1 reduced the accumulation of AML cells in S-phase with gemcitabine or cytarabine treatment.

A phase II study of gemcitabine as a single agent demonstrated no significant activity in relapsed and refractory childhood acute leukemia.\textsuperscript{55} However, these results were not anticipated and the lack of activity was unclear though patients were heavily pre-treated and gemcitabine was administered as a continuous 360 min infusion rather than a more contemporary intermittent schedule with a 30 min to 1 h infusion.

Our preclinical studies suggest that cabazitaxel triggers a mitotic cell cycle arrest which leads to cell death and can significantly prolong survival in multiple murine models of AML. Though pre-clinical studies have shown that paclitaxel and docetaxel were efficient in killing pediatric solid tumors and acute leukemia, tubulin-stabilizing agents have undergone limited clinical testing in pediatric oncology.\textsuperscript{52,53} There is evidence for taxanes exhibiting synergistic toxicity when combined with other cytotoxic agents such as cisplatin in highly resistant brain tumors.\textsuperscript{52} It has been suggested that limited success with taxanes may be attributed, in part, to early phase trials conducted in heavily pre-treated patients.\textsuperscript{52,54} More recently, results from the NCT01751308 clinical trial evaluated the safety and efficacy of cabazitaxel in pediatric patients with refractory solid tumors. Although no objective responses were observed in the phase I dose-escalation portion of the trial, a MTD was established in this pediatric population. The lack of neurotoxicity in this clinical trial is of potential relevance in regard to safety concerns of taxanes in children and allowing for potential clinical translation in a high-risk and/or relapsed/refractory setting.

In this study, we also observed multiple kinase inhibitors targeting a variety of kinases involved in mitosis including alisertib (aurora A; active 4/8), barasertib (aurora B; active 3/8), MK-1775 (Wee1; active 7/8) rigosertib (polo-like kinase; active 7/8), and volasertib (polo-like kinase; active 7/8) and motor protein inhibitor ARRY-520 (Eg5; active 3/8) to have anti-leukemic activity, highlighting a vulnerability inherent to AML, among other cancers (Supplementary Data 2). Several of these compounds are in late stage clinical development, specifically polo-like kinase 1 inhibitors, which demonstrated broad activity in both our HTS and in primary patient samples and have been recognized as Innovative Therapy in Leukemia by the FDA; volasertib has been designated an orphan drug and received breakthrough therapy status for the treatment of AML by the FDA.\textsuperscript{55,56} However, a primary analysis of a phase III study for the treatment of AML with volasertib plus low-dose cytarabine (LDAC) versus placebo plus LDAC did not meet the primary endpoint and patients receiving volasertib plus LDAC were at higher risk for fatal infections.\textsuperscript{57} Therefore, further work will be required to refine our understanding of cell cycle regulation and aberrant function of key regulators that may have a broad role in leukemogenesis or that may associate with specific subtypes and may guide our selection of the optimal mitotic-targeted agent for the treatment of AML.

The clinically used JAK inhibitor ruxolitinib demonstrated selective activity for AMKL and had slightly greater activity compared to tofacitinib. Interestingly, we found a significantly higher expression of STAT5A in AMKL compared to non-AMKL cell lines, including those with FLT3-ITD; and expression highly correlated with ruxolitinib sensitivity compared to other JAK-STAT family members. Our findings suggest that targeting STAT5A is integral to the underlying mechanism driving ruxolitinib’s selective activity in AMKL. While ruxolitinib has been approved for intermediate to high-risk myelofibrosis, the Children’s Oncology Group has completed a phase 1 study in relapse/refractory solid tumors, leukemia, and myeloproliferative neoplasms where a tolerable pediatric dose and schedule was established;\textsuperscript{10} current clinical trials are evaluating ruxolitinib in combination with chemotherapy for the treatment of pediatric lymphoblastic leukemia (NCT02723994).

Collectively, these data provide a rationale for the evaluation of gemcitabine and cabazitaxel in pediatric AML. These agents demonstrated broad activity across multiple high-risk subtypes and may exhibit therapeutic benefit in other subtypes of childhood AML. Similarly, our data provide justification for the clinical evaluation of ruxolitinib for the treatment of pediatric AMKL. Further evaluation regarding optimal dose and schedule in addition to the identification of drug combinations will inform future clinical trial design.
Methods

Cell culture and reagents. Human AML cell lines HEL 92.1.7 (HEL; ATCC TIB-180), HL-60 (ATCC CCL-240),Meg-01 (ATCC CRL-2211), MV-4-11 (ATCC CRL-1193) were derived from patients with adult AML and cultured in RPMI 1640 supplemented with 10% FBS and 1 µM hydrocortisone. Primary AML blast cells were used within 7 days of isolation without culture expansion. For cell viability evaluations, HEL cells (2 × 10^5) were used in 96-well plates with 10% FBS and were incubated for 24 h at 37 °C with 5% CO2. M07e cells were supplemented with 10 ng/mL IL-3 (Life Technologies); M-055 cells were obtained with patient or parent/guardian-provided informed consent under protocol M055-04-00, which was approved by the Institutional Review Board of SJCRH. For primary AML blast cell culture, patients provided informed consent; the protocol was approved by the Institutional Review Board of MD Anderson Cancer Center. Primary AML blast cells were cultured in RPMI1640 media with L-glutamine and supplemented with 10% FBS and 1 µM hydrocortisone. AML patient samples were obtained with patient or parent/guardian-provided informed consent under protocol M055-04-00, which was approved by the Institutional Review Board of SJCRH. For primary AML blast cell culture, patients provided informed consent; the protocol was approved by the Institutional Review Board of MD Anderson Cancer Center. Cell viability was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Sigma). Primary AML blast cells were treated with a dose of 100,000 cells/well and treated with increasing concentrations of drug for 72 h. The following criteria were used to determine the minimum effective concentration (MEC): (1) the drug is FDA-approved, (2) the pediatric drug is not currently used clinically or under investigation for the treatment of AML, and (3) the drug is in phase 1 pediatric testing, and (4) the agent is in phase 1A testing.

Cell viability assays. Cell viability was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Sigma-Aldrich) or Cell Titer Glo (Promega) in a low-throughput manner. Cell lines were seeded in 96-well plates and treated with increasing concentrations of drug for 72 h. Three independent experiments were performed (18 total replicates per concentration). At 68 h after treatment, 10 µL of MTT reagent (5 mg/mL MTT in PBS) was added to each well and plates were incubated at 37 °C with 5% CO2 for 4 h, formazan crystals were solubilized with 100 µL of 0.1 M sodium hydroxide (Sigma-Aldrich), and Cell Titer Glo was used according to the manufacturer’s instructions. The absorbance or luminescence was measured using a Synergy H4 (BioTek Instruments, Inc., Winooski, VT). The half maximal inhibitory concentration (IC50) was calculated by nonlinear regression analysis in the software program GraphPad Prism version 5.04 (GraphPad Software, La Jolla, CA).

Ex vivo drug treatments with primary AML blast samples. Bone marrow MSCs were plated in 96-well plates at a density of 10,000 cells/well in complete MSC media. After 48 h, complete MSC media was removed and replaced by RPMI1640 with L-glutamine supplemented with 10% FBS and 1 µM hydrocortisone (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO). A total of 5 × 10^4 primary AML blast cells were added to each well and treated with increasing concentrations of single agent (6 concentration points; 10-fold dilution). After 96 h treatment, primary AML blast cells were harvested and washed with PBS and counted using a hemocytometer. Cell viability was determined using the Cell Titer Glo assay (Promega) and expressed as percentage of viability compared to untreated controls for each drug concentration.

Cellular uptake and accumulation studies. Logarithmically growing cells (3 × 10^5) were washed with PBS and seeded in 12-well plates. DMSO solution containing a mixture of unlabeled and radioactively labeled cytarabine (total concentration 1, 10, or 100 µM) or gemcitabine (total concentration 1 µM) and were incubated for 5–120 min at 37 °C with 5% CO2. H1-cytarabine (15 Ci mmol−1) and H2-gemcitabine (162.6 Ci mmol−1) were purchased from Moravek Biochemicals (Brea, CA, USA). Cells were washed twice with ice-cold PBS; cell pellets were resuspended in 400 µL 1 N NaOH and incubated (300 rpm) at room temperature for 2 h. Then samples were neutralized with 200 µL 1 M HCl; a 25 µL aliquot of lysis was used to estimate protein concentration using a Pierce BCA protein assay kit (Thermo Scientific). Total radioactivity was measured by a Tri-Carb 4810TR liquid scintillation counter (Perkin-Elmer) after mixing the sample with 4 mL of Emulsifier Safe (Perkin Elmer); the results were normalized to total protein content as measured by a Pierce BCA protein assay kit. Three independent experiments were performed in triplicate.

Total radioactivity was also detected in the nuclear cell fraction, for these experiments 5 × 10^6 cells were treated with a mixture of unlabeled and radioactively labeled cytarabine or gemcitabine (total concentration 1 µM) for 2 h, washed twice with ice-cold PBS then resuspended in 500 µL 1× hypotonic buffer (20 mM Tris–HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl2) and incubated for 15 min on ice. Next, 25 µL of detergent (10% NP-40, Thermo Scientific) was added, then samples were vortexed for 10 s at highest setting followed by centrifugation for 10 min at 3000 × g. The nuclear pellet was resuspended in 50 µL complete extraction buffer (cell extraction buffer [Life Technologies], protease inhibitor cocktail [Calbiochem], 1 mM PMSF) and incubated for 30 min on ice with vortexing at 10 min intervals then centrifuged for 30 min at 14,000× g at 4 °C. The supernatant containing the nuclear fraction was filtered to a clean tube; 40 µL was used to determine radioactivity using the liquid scintillation counter. A Pierce BCA protein assay was performed on the cytoplasmic fraction of each sample and results were normalized to total protein content. Three independent experiments were performed in triplicate.

HPCL to detect compounds and metabolites. Logarithmically growing cells (3 × 10^5) were washed and seeded in 12-well plates with serum-free medium containing a mixture of unlabeled and radioactively labeled cytarabine or gemcitabine (total concentration 1 µM) for 2 h, washed twice with ice-cold PBS then resuspended in 500 µL of buffer containing 70% methanol and 30% 15 mM Tris (pH 7.4) and shaken (300 rpm) for 10 min at 4 °C. After centrifugation, 30 µL of cell extract was removed for protein concentration measurement using a Pierce BCA protein assay kit. Intracellular cytarabine, gemcitabine, and phosphorylated metabolites were isolated according to the following standards were used: Ara-C, cytidine 5′-monophosphate disodium salt (Sigma), cytidine 5′-diphosphocholine sodium salt (Sigma), cytidine 5′-diphosphate trisodium salt (Sigma), cytidine 5′-triphosphate disodium salt (Sigma), gemcitabine hydrochloride (Sigma), gemcitabine monophosphate (Toronto Research Chemicals), gemcitabine diphasphate trimethylamine salt (Toronto Research Chemicals), gemcitabine triphosphate diethylamine (Toronto Research Chemicals). All standards were prepared to a concentration of 1 mg mL−1 in water. Analysis was carried out on a Waters Alliance HPLC system with a Phenomenex Luna C18 column (5 µm, 250 × 4.6 mm, 3 µL particles) at 30 °C. Mobile phase A was 500 mM NH42HPO4 and mobile phase B was 500 mM NH42H2PO4, pH 3.4. Flow rate is varied from 0.5 to 1.0 mL min−1 at a temperature of 40 °C. The gradient was modified in

NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-019-09917-0 | www.nature.com/naturecommunications

13
such a manner that it is held isocratically from 0 to 10 min at a flow rate of 0.5 mL min$^{-1}$. At 10 min the flow changes to 1.0 mL min$^{-1}$ and a linear gradient runs from 0% to 100% B from 10.01 to 40.0 min. Fractions were collected directly into scintillation vials at 1 min intervals using an Eldex Universal Fraction Collector. The total run time was 70 min. After addition of Scintisafe 30% scintillation fluid (Perkin Elmer), samples were vortexed and radioactivity was measured on a Tri-Carb 460TR liquid scintillation counter. Total radioactivity was expressed as disintegrations per minute (DPMs) and normalized to protein concentration.

Three independent experiments were performed.

**Silencing of ENT1 and OCTN1 expression.** The on-target plus SMART pool human SLC22A4 siRNA for OCTN1 silencing (Dharmacon, Inc./Horizon, Cambridge, UK), and the Mission siRNAs targeting ENT1 or negative non-targeting control (Sigma-Aldrich) were used in all experiments. MOLM-13 cells were transfected with siRNA using siRNA OPTIN (or siRNA ENT1 using Nuclear-actor II and Nucleofector Kit C (Lonza) program X-001, according to the manufacturer’s protocol. Briefly, MOLM-13 cells (3 × 10$^6$) were transfected with 200–500 nM siRNA for each sample; suppression of OCTN1 (Hs00266820_m1) and ENT1 (Hs01085704_m1) was evaluated using a TaqMan qRT-PCR assay at 48 h post-transfection; expression levels were normalized to GAPDH (Hs02758991_g1; VIC). Cytotoxicity studies were initiated in MOLM-13 cells 24 h after transfection. Asynchronous cells were treated with PBS, 10 nM gemcitabine, or 100 nM cytarabine for 16 h; then cells were harvested for cell cycle distribution as described below.

**Cell cycle distribution and induction of mitosis.** Asynchronous cells were treated with 5 or 50 µM cabazitaxel for up to 24 h; DMSO was the control agent. At indicated time points (4, 8, 12, 16, 24 h) cells were collected and washed once with 0.1% EDTA (Rica Chemical Company, Arlington, TX) in PBS then washed once with PBS only. Next, cells were fixed with ice-cold 70% ethanol for 30 min on ice or stored at −20°C for up to 1 month. Cells were spun down at 450 g for 10 min and stained with DAPI (DAPI final concentration 1 µg mL$^{-1}$) ([ThermoFisher Scientific](#)) in 0.1% Triton-X [ThermoFisher Scientific](#)/PBS for 30 min at room temperature and protected from light. The DNA content was determined using a BD LSR II flow cytometer (BD Biosciences, San Jose, CA, USA). The cell cycle distribution was analyzed using FlowJo v10.0.08 software (FlowJo, LLC, Ashland, OR).

For biparametric analysis of mitosis, cells were fixed with 16% paraformaldehyde (Avantor, Center Valley, PA) in PBS at room temperature for 10 min, washed twice with PBS then permeabilized with ice-cold 95% methanol ([ThermoFisher Scientific](#)) and incubated at −20°C. Cells were rehydrated in FACS buffer (PBS + 4% FBS) and stained with cyclin B1-APC (Cell Signaling Technology, Inc., Danvers, MA) and phospho-histone H3-FITC (Cell Signaling Technology, Inc.). Two-color flow cytometry was performed using a BD LSR II flow cytometer (BD Biosciences) and the data was analyzed using FlowJo (FlowJo, LLC).

**Binding assay.** Binding of ruxolitinib and tofacitinib to purified JAK1, JAK2, JAK3, and TYK2 kinases were performed using a commercially available KdELECT assay (DiscoverRx, Fremont, CA), as previously described. The binding constant (K_d) was calculated with a standard dose-response curve using the Hill equation (slope set to −1); curves were fitted using a non-linear least square fit with the Levenberg–Marquardt algorithm.

**Kinase assay.** In vitro profiling of JAK1, JAK2, JAK2V617F, JAK3, and TYK2 kinase were performed at Reaction Biology Corporation. Briefly, specific kinase/substrate pairs were prepared in fresh base reaction buffer (20 mM HEPES at pH 7.5, 10 mM MgCl$_2$, 0.1 mM EGTA, 0.02% Brij35, 0.02 mg/mL BSA, 0.1 mM Na$_2$VO$_3$, 2 mM DTT, 1% DMSO). Compounds were delivered into the reaction and incubated for 20 min. Next, 33p-ATP was delivered to the reaction mixture to initiate the reaction and incubated for 2 h at room temperature. Reactions were spotted onto P81 ion exchange paper and kinase activity was detected by filter-binding method.

**RNA isolation and qRT-PCR.** RNA was isolated from cell lines using Trizol (Invitrogen) chloroform (Sigma-Aldrich) extraction. cDNA was generated from 1 µg of RNA using the SuperScript IV First-Strand Synthesis System (Invitrogen) with chloroform (Sigma-Aldrich) extraction. cDNA was generated from 1 µg of RNA using TruSeq RNA Prep v2 kits according to the manufacturer’s instructions, at the SJCRH Hartwell Center. To quantify the expression level of each gene, we performed the following procedures: (1) for each amplified cDNA, we obtained the average of three replicates; due to the high variations of coverage across different exons for each gene, we used the average coverage of the best covered exon as the expression level for each gene. After we combine the expression for all 12 samples, we only retained those genes whose expression level is at least 10 in at least one sample in order to exclude genes that are underexpressed or poorly expressed at all samples. For gene expression comparisons, we obtained counts of the number of reads per gene and carried out fragment per kilobase mapped (FPKM) normalization and a quantile normalization was performed to adjust different sequencing depths for each sample.

**Murine bone marrow transplantation and vaccination.** All animal studies were performed in accordance with Animal Care and Use Programs under protocols approved by the Institutional Animal Care and Use Committee at SJCRH or The Ohio State University (OSU). We have complied with the relevant ethical considerations for animal research overseen by this committee. Bone marrow from 4–6-week-old female C57BL/6 mice was harvested, lineage depleted (Lineage Cell Depletion Kit, Miltenyi Biotec Inc., Auburn, CA) and cultured in the presence of cytokines for 24 h prior to transduction on RetroNectin (Takara Bio Inc., Kyoto, Japan). Ectopic envelope-pseudotyped retroviral vectors were produced and replication-incompetent supernatant was made by transiently transfecting 293 T cells. For primary bone marrow transplants, 0.1 × 10$^6$ single (GFP, mCherry) or double-positive cells with 1 × 10$^6$ protected cells were injected intravenously via tail vein on day 0. Recipient mice were conditioned with total body irradiation at a dose of 1000 rad divided on days 0–2. For secondary transplants, 2 × 10$^6$ bone marrow and spleen cells from morbld mice were isolated and injected intravenously into recipient mice conditioned with 500 rad. Alternatively, double-positive cells isolated from secondary transplants and were transduced with a luciferase-BFP to generate CG/V617F-Luc + AMKL blast; GFP/mCherry/BFP positive cells were purified by flow cytometry then injected into conditioned tertiary or quaternary recipients.

**Establishment of in vivo gemcitabine maximum tolerated dose.** All animal studies were performed in accordance with Animal Care and Use Programs under protocols approved by the Institutional Animal Care and Use Committee at OSU. We have complied with the relevant ethical considerations for animal research overseen by this committee. Mice were housed in a barrier facility. Non-tumor bearing CG-V617F-Luc (Boyj) and 3% 200 µL fetal bovine serum (FBS) and stained with cyclin B1-APC (Cell Signaling Technology, Inc., Danvers, MA) and phospho-histone H3-FITC (Cell Signaling Technology, Inc.). Two-color flow cytometry was performed using a BD LSR II flow cytometer (BD Biosciences) and the data was analyzed using FlowJo (FlowJo, LLC).

**In vivo efficacy studies in murine models of AML.** All animal studies were performed in accordance with Animal Care and Use Programs under protocols approved by the Institutional Animal Care and Use Committee at SJCRH or The Ohio State University (OSU). We have complied with the relevant ethical considerations for animal research overseen by this committee. Mice were housed in a barrier facility. For cell line xenografts, NSG mice were procured from the OU Comprehensive Cancer Center Target Validation Shared Resource. To label CHRF288-11 cells, lentivirus encoding firefly luciferase (hLuc2-luc) and YFP (CRL-2740) were co-packaged with VSV-G envelope (SJRH Vector Core) was used. Briefly, CHRF288-11 cells were transduced by spinfection using retroinfecton coated plates and 10 µL of concentrated viral supernatant obtained from the SJCHR Vector Core. Following transduction, cells were single cell sorted into each well of a 96-well plate by flow cytometry, individual clones were expanded, then evaluated in vivo for engraftment. For efficacy studies, mice were injected intravenously with tail vein with 5 × 10$^6$ CHRF288-11-LUC/YFP+ or ML-2-LUC/YFP+ cells; engraftment was monitored by noninvasive imaging performed once weekly starting on day 6 or 7 after injection. The Luciferase substrate D-luciferin firefly potassium salt (Gold Biotechnology, Inc., St. Louis, MO, USA) was dosed by intraperitoneal injection at a dose of 20 mg/mL. Animals were anesthetized by 1.5−2.5% isoflurane (Baxter, Deerfield, IL, USA) inhalation; bio-luminescence was determined 5 min later using a Xenogen IVIS-200 imaging system (Perkin Elmer). Total body bioluminescence was quantified for the body area that included each mouse in its entirety (Living Image 4.3.1, Perkin Elmer). For AMKL, PDX, 1 × 106 cells whole bone marrow and spleen cells isolated from an individual primary transplant recipient were injected intravenously into sublethally irradiated (200 rad) NSG-SGM3 female mice that were 8–12 weeks old for expansion. For efficacy studies, 1 × 106 cells whole bone marrow and spleen cells isolated from an individual secondary transplant recipient were injected intravenously into sublethally irradiated (200 rad) NSG-SGM3 female mice that were 8–12 weeks old. Transplant studies used whole bone marrow and spleen cells from an individual MIP2TD/WT*FL5172V1ITD double knock-in (dkI) mouse or individual CG/V617F-Luc+ tertiary transplant recipient that had been stored in liquid nitrogen. After thawing, 2 × 10$^6$ cells were injected intravenously into sublethally irradiated (200 rad) NSG-SGM3 female mice that were 8–12 weeks old. Transplant studies used whole bone marrow and spleen cells from an individual MIP2TD/WT*FL5172V1ITD double knock-in (dkI) mouse or individual CG/V617F-Luc+ tertiary transplant recipient that had been stored in liquid nitrogen.
peripheral blood for the remainder of the study. To establish the PDX model, a single vial of patient cells was thawed and engrafted in primary recipients then further expanded in secondary recipients; bone marrow and spleen cells were stored in liquid nitrogen. On day 1, 8–12-week-old triple transgenic NGS-SGM3 mice (The Jackson Laboratory; Bar Harbor, ME, USA) expressing human IL-3, GM-CSF, and SCF were sublethally irradiated (200 rad) with one dose of total body irradiation. On day 0, 2 × 10⁶ cells were injected intravenously via tail vein into tertiary recipients. Leukemia progression was monitored by white blood cells (WBC) count and/or detection of Ly5.2+ cells. GFP/mCherry-double positive, or human CD45 positive cells in peripheral blood by flow cytometry weekly. All mice were observed daily and humanely euthanized when showing signs of progressive disease including, hind limb paralysis, weight loss more than 15%, and lethargy.

To evaluate anti-leukemic activity NSG/NSG-SGM3 mice and dKi AML primary recipients were randomly assigned upon detection of significant tumor burden, based on imaging data or peripheral blood analysis, to receive vehicle, cytarabine or gemcitabine 50 mg·kg⁻¹, or cabazitaxel 5 mg·kg⁻¹ every 4 days for 3 weeks; some mice were administered two cycles of dosing regimen with 1 week off between cycles as indicated. CG-V617-Luc+ quaternary recipients and dKi secondary recipients were administered 50 or 100 mg·kg⁻¹ cytarabine once daily for 5 days, 3 mg·kg⁻¹ gemcitabine once daily for 5 mg·kg⁻¹ gemcitabine once every 4 days for 3 weeks, some mice were administered two cycles of dosing regimen with 2 weeks off between daily schedules and 1 week off for the intermittent schedule. Cytarabine and gemcitabine were formulated in PBS; cabazitaxel was made fresh daily, formulated in 5% ethanol/5% Tween-80/5% glucose water; all treatments were administered by intraperitoneal injection. Ruxolitinib was reconstituted in 0.5% methylcellulose with overnight end-over-end mixing and administered by oral gavage twice daily for 5 days for 4 weeks. Treatment started on day 10 (CHRF288-11-LUC/YFP+ or 17 (ML-2 LUC/YFP+)) when significant bone marrow engraftment was observed, as determined by imaging analysis. Similarly, treatment started on day 13 for mice primary recipients with dKI AML or day 7 for secondary recipients, and day 17 or 20 for CG-V617-Luc+ AMKL, after first observation of ≤2% Ly5.2+ or GFP+ mCherry-double+ cells in peripheral blood or spleen. Treatment started on day 11 for the AMKL. PKD after first observation of >2% human CD45 cells in peripheral blood.

Bone marrow and spleocytes were isolated from mice transplanted with dKi AML and CG-V617 AMKL. Following red blood cells lysis, dKi AML leukemic blasts from vehicle treated mice were evaluated for accumulation of cytarabine and gemcitabine using the uptake assay described above. CG-V617 AMKL leukemic blasts from treatment-naive tertiary transplants were subjected to red blood cell lysis then subjected to cell viability assay using increasing concentrations of ruxolitinib as described above.

Pharmacokinetics of ruxolitinib. Animal studies were performed under protocols approved by the Institutional Animal Care and Use Committee at OSU. Single dose pharmacokinetic studies were conducted in 8–12-week-old female and male NSG and BoyJ mice to determine a ruxolitinib dose that produces human equivalent exposure. For these studies, 60 mg·kg⁻¹ of ruxolitinib was administered by oral gavage in 0.5% methylcellulose. Serial blood collection was performed from 15 min up to 240 min after dose administration. Total ruxolitinib concentrations in plasma were measured using a modification of previously published methods. Quantitation was carried out by liquid chromatography-tandem mass spectrometry with a Vanquish UHPLC system and a TSQ Quantum mass spectrometer (ThermoFisher Scientific); separation was achieved in 5 min using an Accucore Vanquish C18 column and the system was controlled using Thermo Trace Finder General Quan software.

Immunoblot analysis. For protein expression of p-STAT5A and total-STAT5A, cells were lysed and whole cell lysates were prepared using radioimmunoprecipitation assay buffer (Sigma-Aldrich) supplemented with protease and phosphatase inhibitors. Total cell lysate (20 µg) was separated by SDS-PAGE and transferred to PVDF membranes. Western blot analysis was then performed using p-STAT5A (1:1000, Cell Signaling Technology, Danvers, MA) and total-STAT5A (1:1000, Cell Signaling Technology); secondary α-rabbit or α-mouse antibodies (1:2000, Jackson ImmunoResearch, West Grove, PA) were used and proteins were visualized using the SignalFire ECL Reagent (Cell Signaling Technology) on an Odyssey FC Imaging System (LI-COR, Lincoln, NE). GAPDH (Santa Cruz Biotechnology, Inc., Dallas, TX) was used a loading control; immunoblots were performed a minimum of three times on samples collected from different experiments. Uncropped and unprocessed blots are provided in the Data Source file.

Statistical analysis. Prism software (GraphPad Software) was used for statistical analyses. Kaplan–Meier analysis of animal survival and statistical significance of data was determined by log rank test; P < 0.05 was considered statistically significant. All other statistical tests performed are indicated in corresponding text or figure legend; P < 0.05 was considered statistically significant. A linear regression analysis was performed to evaluate a goodness of fit and determine the Pearson correlation between STAT5A expression and ruxolitinib sensitivity.

Data availability

The RNA-seq data generated and analyzed in this study are available at the Gene Expression Omnibus ( GEO) repository of the National Center for Biotechnology Information under accession code GSE126489. The authors declare that all data generated from this study are included in this publication and its Supplementary Information, Source Data file (Fig. 2–7; Supplementary Figs. 1, 3, 5, 7, 9, 13, 15, 16), or available from the corresponding author on request.

Code availability

High-throughput screening data was analyzed using our in-house Robust Interpretation of Screening Experiments (RISE) application written in Pipeline Pilot (Accelrys, v8.5) and the R program (R Development Core Team). The Pipeline Pilot protocol (exported as a Pipeline Pilot formatted xml file), including the embedded R code, are reported in Supplementary Software.

Received: 6 June 2018 Accepted: 5 April 2019
Published online: 16 May 2019

References

1. Ribero, R. C. Advanced treatment of de-novo pediatric acute myeloid leukemia. *Curr. Opin. Oncol.* 26, 656–662 (2014).
2. Rubnitz, J. E. & Inaba, H. Childhood acute myeloid leukaemia. *Br. J. Haematol.* 159, 259–276 (2012).
3. Athale, U. H. et al. Biology and outcome of childhood acute megakaryoblastic leukaemia: a single institution’s experience. *Blood* 97, 3727–3732 (2001).
4. Barnard, D. R. et al. Comparison of childhood myelodysplastic syndrome, AML FAB M6 or M7, CCG-2891: report from the Children’s Oncology Group. *Pediatr. Blood Cancer* 49, 17–22 (2007).
5. Chen, Y. et al. Prognostic significance of 11q23 aberrations in adult acute myeloid leukaemia and the role of allogeneic stem cell transplantation. *Leukemia* 27, 836–842 (2013).
6. Paganó, L. et al. Acute megakaryoblastic leukaemia: experience of GIMEMA trials. *Leukemia* 16, 1622–1626 (2002).
7. Thiede, C. et al. Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukaemia: association with FAB subtypes and identification of subgroups with poor prognosis. *Blood* 99, 4326–4332 (2002).
8. Barretina, J. et al. The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature* 483, 603–607 (2012).
9. Garnett, M. J. et al. Systematic identification of genomic markers of drug sensitivity in cancer cells. *Nature* 483, 570–575 (2012).
10. Grossmann, V. et al. High incidence of RAS signalling pathway mutations in DLL-rearranged acute myeloid leukaemia. *Leukemia* 27, 1933–1936 (2013).
11. Tsato, V., Voltan, R., Gonelli, A., Secchierno, P. & Zauli, G. MDM2/X inhibitors under clinical evaluation: perspectives for the management of hematological malignancies and pediatric cancer. *J. Hematol. Oncol.* 10, 133 (2017).
12. Wen, Q. et al. Identification of regulators of polyploidization presents therapeutic targets for treatment of AMKL. *Cell* 150, 575–589 (2012).
13. Xie, C. et al. Panobinostat enhances cytarabine and daunorubicin sensitivities in AML cells through suppressing the expression of BRCA1, CHK1, and Rad51. *PloS One* 8, e79106 (2013).
14. Cizmaz, C. M., Kim, D. H. & Sachs, Z. The role of the proteasome in AML. *Blood Cancer J.* 6, e503 (2016).
15. Ciccolini, J., Serdejbi, C., Peters, G. J. & Giovanetti, E. Pharmacokinetics and pharmacodynamics of Gemcitabine as a maintenance in adult and pediatric oncology: an EORTC-PAMM perspective. *Cancer Chemother. Pharmacol.* 78, 1–12 (2016).
16. Reid, J. M. et al. Phase I trial and pharmacokinetics of gemcitabine in children with advanced solid tumors. *J. Clin. Oncol.* 22, 2445–2451 (2004).
17. Wiley, J. S., Jones, S. P. & Sawyer, W. H. Cytosine arabinoside transport by human leukemic cells. *Eur. J. Cancer Clin. Oncol.* 19, 1067–1074 (1983).
18. Jordheim, L. P., Durantel, D., Zoulim, F. & Dumontet, C. Advances in the development of nucleoside and nucleotide analogues for cancer and viral diseases. *Nat. Rev. Drug Discovery* 12, 447–464 (2013).
19. Obata, T., Endo, Y., Murata, D., Sakamoto, K. & Sasaki, T. The molecular landscapes of antitumor 2′-deoxyxycytidine analogues. *Curr. Drug Targets* 4, 305–313 (2003).
20. Drenberg, C. D. et al. OCTIN1 is a high-affinity carrier of nucleoside analogues. *Cancer Res.* 77, 2102–2111 (2017).
21. Pastor-Anglada, M., Cano-Soldado, P., Errasti-Murugarren, E. & Casado, F. J. SLC28 genes and concentrative nucleoside transporter (CNT) proteins. *Nucleosides Nucleotides Nucleic Acids* 38, 972–994 (2008).

22. Radzicka, A. & Wolfsdend, R. A proficient enzyme. *Science* 267, 90–93 (1995).

23. Vivian, D. & Polli, J. E. Mechanistic interpretation of conventional Michaelis–Menten parameters in a transporter system. *Eur. J. Pharm. Sci.* 64, 44–52 (2014).

24. Ewald, B., Sampath, D. & Plunkett, W. Nucleoside analogs: molecular mechanisms signaling cell death. *Oncogene* 27, 6522–6537 (2008).

25. Karjalainen, R. et al. JAK1/2 and BCL2 inhibitors synergize to counteract bone marrow stromal cell-induced protection of AML. *Blood* 130, 789–802 (2017).

26. Jordan, M. A. & Wilson, L. Microtubules as a target for anticancer drugs. *Nat. Rev. Cancer* 4, 233–269 (2004).

27. Gruber, T. A. et al. An Inv(16)(p13.3q24.3)-encoded CBFA2T3-GLIS2 fusion protein defines an aggressive subtype of pediatric acute megakaryoblastic leukemia. *Cell Cancer Cell* 22, 683–697 (2012).

28. de Rooij, J. D. et al. Pediatric non-Down syndrome acute megakaryoblastic leukemia is characterized by distinct genomic subsets with varying outcomes. *Nat. Genet.* 49, 431–456 (2017).

29. Dang, J. et al. AMKL chimeric transcription factors are potent inducers of leukemia. *Leukemia* 31, 2228–2234 (2017).

30. Gruber, T. A. & Downing, J. R. The biology of pediatric acute megakaryoblastic leukemia. *Blood* 126, 943–949 (2015).

31. Drenberg, C. D. et al. Evaluation of artemisinins for the treatment of acute myeloid leukemia. *Pharmacol. Ther.* 77(1), 1231–1243 (2016).

32. Bernot, K. M. et al. Eradicating acute myeloid leukemia in a MLL(PTD/wt)Flt3 (ITD/wt) murine model: a path to novel therapeutic approaches for human disease. *Blood* 122, 3778–3783 (2013).

33. Zorno, N. A. et al. MLL partial tandem duplication and Flt3 internal tandem duplication in a double knock-in mouse recapitulates features of counterpart human acute myeloid leukemias. *Blood* 120, 1130–1136 (2012).

34. Changelian, P. S. et al. Prevention of organ alloraft rejection by a specific Janus kinase 3 inhibitor. *Science* 302, 875–878 (2003).

35. Quintas-Cardama, A. et al. Preclinical characterization of the selective JAK1/2 inhibitor INC018424: therapeutic implications for the treatment of myeloproliferative neoplasms. *Blood* 115, 3109–3117 (2010).

36. Beloukhait, K. et al. Generation of three layers vascular wall by using BMP2-treated MSC involving HIF-1alpha and Id1 expressions through JAK/STAT pathways. *Stem Cell Rev.* 7, 847–859 (2011).

37. Wiithuhn, B. et al. JAK2 associates with the erythropoetin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoetin. *Cell* 74, 227–236 (1993).

38. Besanconot, R. et al. JAK2 and MPL protein levels determine TPO-induced megakaryocyte proliferation vs differentiation. *Blood* 124, 2104–2115 (2014).

39. Kauhansky, K. Historical review: megakaryopoiesis and thrombopoiesis. *Blood* 111, 981–986 (2008).

40. Loh, M. et al. A phase 1 dose finding study of ruxolitinib in children with relapsed or refractory solid tumors, leukemias, or myeloproliferative neoplasms: A Children’s Oncology Group phase 1 consortium study (ADVL1011). *Pediatr. Blood Cancer* 62, 1717–1724 (2015).

41. Ravandi, F., Kantjarjian, H., Giles, F. & Cortes, J. New agents in acute myeloid leukemia and other myeloid disorders. *Cancer* 100, 441–454 (2004).

42. Shanks, R. H., Rizzieri, D. A., Flowers, J. L., Colvin, O. M. & Adams, D. J. Preclinical evaluation of gemcitabine combination regimens for application in acute myeloid leukemia. *Clin. Cancer Res.* 11, 4225–4233 (2005).

43. Wang, E., Gulbis, A., Hart, J. & Nieto, Y. The emerging role of gemcitabine in conditioning regimens for hematopoietic stem cell transplantation. *Biol. Blood Marrow Transplant.* 20, 1382–1389 (2014).

44. Plunkett, W., Huang, P., Seary, C. E. & Gandhi, V. Gemcitabine: preclinical pharmacology and mechanisms of action. *Semin. Oncol.* 23, 3–15 (1996).

45. Peters, G. J. et al. Basis for effective combination cancer chemotherapy with antimetabolites. *Pharmacol. Ther.* 87, 227–253 (2000).

46. Bouffard, D. Y. & Momparler, R. L. Comparison of the induction of apoptosis in human leukemic cell lines by 2′,2′-difluorodeoxycytidine (gemcitabine) and cytosine arabinoside. *Leuk. Res.* 19, 849–856 (1995).

47. Gandhi, V. & Plunkett, W. Modulatory activity of 2′,2′-difluorodeoxycytidine on the phosphorylation and cytotoxicity of arabinosyl nucleosides. *Cancer Res.* 50, 3675–3680 (1990).

48. Hertel, L. W. et al. Evaluation of the antitumor activity of gemcitabine (2′,2′-difluoro-2′-deoxycytidine). *Cancer Res.* 50, 4417–4422 (1990).

49. Drenberg, C. D. et al. ABC44 is a determinant of cytotoxicity in preclinical setting. *Clin. Transl. Sci.* 9, 51–59 (2016).

50. Adema, A. D. et al. Overexpression of MRP4 (ABCC4) and MRP5 (ABCC5) confer resistance to the nucleoside analogs cytarabine and treosulfan, but not gemcitabine. *SpringerPlus* 3, 126 (2014).

51. Angiolillo, A. L. et al. Phase II study of gemcitabine in children with relapsed acute lymphoblastic leukemia or acute myelogenous leukemia (ADVL0022): a Children’s Oncology Group Report. *Pediatr. Blood Cancer* 46, 193–197 (2006).

52. Andre, N. & Meille, C. Taxanes in paediatric oncology: and now? *Cancer Treat. Rev.* 32, 65–73 (2006).

53. Reynolds, C. P. et al. Initial testing (stage 1) of the anti-microtubule agents cabazitaxel and docetaxel, by the pediatric preclinical testing program. *Pediatr. Blood Cancer* 62, 1897–1905 (2015).

54. Woo, M. H. et al. Phase I targeted systemic exposure study of paclitaxel in children with refractory acute leukemias. *Clin. Cancer Res.* 5, 543–549 (1999).

55. Dohner, H. et al. Randomized, phase 2 trial of low-dose cytarabine with or without volasertib in AML patients not suitable for induction therapy. *Blood* 124, 1426–1433 (2014).

56. Gjertsen, B. T. & Schoffski, P. Discovery and development of the Polo-like kinase inhibitor volasertib in cancer therapy. *Leukemia* 29, 11–19 (2015).

57. Dohner, H. et al. Phase III randomized trial of volasertib plus low-dose cytarabine (LDAC) versus placebo plus LDAC in patients with LDAC in patients aged ≥65 years with previously untreated AML, ineligible for intensive therapy. *Haematologica* 101, 185 (2016).

58. Davis, M. I. et al. Comprehensive analysis of kinase inhibitor selectivity. *Nat. Biotechnol.* 29, 1046–1051 (2011).

59. Anastassiadis, T., Deacon, S. W., Devarajan, K., Ma, H. & Peterson, J. R. Comprehensive assay of kinase catalytic activity reveals features of kinase inhibitor selectivity. *Nat. Biotechnol.* 29, 1039–1045 (2011).

60. Soneoka, Y. et al. A transient three-plasmid expression system for the production of high titer retroviral vectors. *Nucleic Acids Res.* 35, 628–633 (2007).

61. Veeraraghavan, S. et al. Simultaneous quantification of ruxolitinib and nilotinib in rat plasma by LC-MS/MS: application to a pharmacokinetic study. *J. Pharm. Biomed. Anal.* 94, 125–131 (2014).