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Original Research

High-throughput drug screening reveals Pyrvinium pamoate as effective candidate against pediatric *MLL*-rearranged acute myeloid leukemia

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ABSTRACT

Pediatric *MLL*-rearranged acute myeloid leukemia (AML) has a generally unfavorable outcome, primarily due to relapse and drug resistance. To overcome these difficulties, new therapeutic agents are urgently needed. Yet, implementing novel drugs for clinical use is a time-consuming, laborious, costly and high-risk process. Therefore, we applied a drug-repositioning strategy by screening drug libraries, comprised of >4000 compounds that are mostly FDA-approved, in a high-throughput format on primary *MLL*-rearranged AML cells. Here we identified pyrvinium pamoate (pyrvinium) as a novel candidate drug effective against *MLL*-rearranged AML, eliminating all cell viability at <1000 nM. Additional screening of identified drug hits on non-leukemic bone marrow samples, resulted in a decrease in cell viability of ~50% at 1000 nM pyrvinium, suggesting a therapeutic window for targeting leukemic cells specifically. Validation of pyrvinium on an extensive panel of AML cell lines and primary AML samples showed comparable viabilities as the drug screen data, with pyrvinium achieving IC_{50} values of <80 nM in these samples. Remarkably, pyrvinium also induced cell toxicity in primary *MLL-AF10*$^{+}$ AML cells, an *MLL*-rearrangement associated with a poor outcome. While pyrvinium is able to inhibit the Wnt pathway in other diseases, this unlikely explains the efficacy we observed as β-catenin was not expressed in the AML cells tested. Rather, we show that pyrvinium co-localized with the mitochondrial stain in cells, and hence may act by inhibiting mitochondrial respiration. Overall, this study shows that pyrvinium is highly effective against *MLL*-rearranged AML *in vitro,* and therefore represents a novel potential candidate for further studies in *MLL*-rearranged AML.

Introduction

The outcome of pediatric Acute Myeloid Leukemia (AML) has improved steadily over the past decades, with current long-term overall survival rates of over 70%. This improvement has mainly been achieved due to better risk stratification, salvage at relapse, and improvements in allogeneic hematopoietic stem cell transplantation and supportive care [1–5].

Risk stratification of this heterogeneous disease is currently performed using early response to therapy (measured as minimal residual disease [MRD]) and genetics, including cytogenetic and molecular features [6]. One of the most common recurrent genetic abnormalities are chromosomal translocations of the *MLL* gene (also known as *KMT2A*). Although clinical outcome for patients with *MLL*-rearranged AML is dependent on the *MLL* translocation fusion partner gene, the prognosis is generally unfavorable. While patients carrying the *MLL-AF9* fusion show event-free survival (EFS) rates of 77%, patients harboring *MLL-AF6, MLL-AF4*, and *MLL-AF10* translocations fare significantly worse, with EFS rates of only 11%, 29%, and 32%, respectively. Overall, pediatric *MLL*-rearranged AML had a 5-year EFS of 44%, and a 5-year overall survival of 56% in a global study published in 2009 [7].

The majority of children with AML receive four or five courses of intensive chemotherapy, mainly based on cytarabine (ara-C) and anthracyclines, such as daunorubicin. Most collaborative group protocols include hematopoietic stem cell transplantation in the first complete remission for selected high risk fusions and/or patients with persistent MRD. This therapeutic framework has remained essentially unchanged for decades. The intensity of therapy is risk-adapted, and is mainly de-

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pendent on the quality of remission following induction therapy [8,9]. However, further intensification of current treatment regimens for high-risk subtypes of pediatric AML is not possible. Hence, to increase the survival rate of AML, additional or novel therapeutic agents are needed to improve the survival for subgroups which experience a poor outcome, such as *MLL*-rearranged AML. Nevertheless, implementing novel drugs for clinical use is an expensive and years-long process with a high failure rate. To avoid these hurdles, the repurposing of drugs already clinically approved for other indications for use in cancer treatment has become an attractive strategy. Such an approach is efficient, economical and allows for rapid identification of candidate drugs suitable for clinical trials [10]. In the present study, we therefore applied a drug repositioning strategy by screening >4000 compounds on primary *MLL*-rearranged AML patient samples using commercially available drug libraries that mostly contain FDA-approved agents. These drug screens identified pyrvinium pamoate (pyrvinium) as an interesting candidate drug for the treatment of *MLL*-rearranged AML, which also demonstrated efficacy against other types of AML.

Materials and methods

Patient samples

Primary AML samples were obtained from the Erasmus MC-Sophia Children's Hospital (Rotterdam, The Netherlands; Supplemental Table 1). Written informed consent was obtained from parents or guardians to use excess of diagnostic material for research purposes, as approved by the Medical Ethics Committee of the Erasmus Medical Center, The Netherlands. A copy of the written consent is available for review by the Editor-in-Chief of this journal on request. Patient characteristics are listed in Supplemental Table 1. For the drug screen, one non-leukemic bone marrow aspirate, namely non-leukemic BM #1, was collected at the Erasmus MC-Sophia Children's Hospital, from a patient with T-cell lymphoma without infiltration in the bone marrow. The other bone marrow aspirate used for the drug screen, namely non-leukemic BM #2, was collected at the Wilhelmina Children's Hospital (Utrecht, The Netherlands), from a patient with Acute Lymphoblastic Leukemia (ALL) in remission at day 79 of the SKION ALL-11 treatment protocol. All samples were processed as described elsewhere [11] and immediately used for viability assays as described below. Briefly, cryopreserved cells were resuspended in culturing medium of RPMI-1640 (Dutch modification, Gibco, Uxbridge, UK) containing 20% FCS (GE healthcare), 100IU/ml penicillin-streptomycin (ThermoFisher), 0.125 µg/ml amphotericin B (ThermoFisher), 0.2 mg/ml gentamycin (ThermoFisher), 5 mg/ml insulin, 5 mg/ml transferrin, 5 ng/ml sodium selenite (ITS; Sigma-aldrich) and 2 mM L-glutamine (ThermoFisher). Note, that sensitivity of cells were not influenced by cryopreservation [12]. Cells were washed in culturing medium twice at 1500 rpm for 5 min at RT. Thereafter, resuspended cells were diluted in culturing medium to a concentration of 1–1.5 × 10^6 cells/ml and incubated at 37 °C in humified air containing 5% CO_2 until further use. The leukemic samples used in this study contained >80% leukemic blasts before and after culture, and the two 'non-leukemic' bone marrow controls contained <1% blasts, as determined by May-Grünwald Giemsa (Merck) stained cytospins.

Cell lines

Human *MLL*-rearranged AML cell lines MV4;11, ML-2, SHI-1, NOMO-1, THP-1, and wildtype *MLL* AML cell lines HEL, KASUMI-1, ME-1, MKPL-1 were all purchased from DSMZ-German collection of microorganisms and cell cultures. The wildtype *MLL* AML cell line CHRF-288–11 was kindly provided by Dr. Gruber (St. Jude Children's Research Hospital, Memphis, TN, USA). Cell line characteristics are listed in Supplemental Table 2. All cell lines were cultured in RPMI-1640 with GlutaMAX (ThermoFisher), 10%–20% fetal calf serum (GE healthcare), 100IU/ml penicillin streptomycin (ThermoFisher), and 0.125 µg/ml amphotericin B (ThermoFisher), at 37 °C in humified air containing 5% CO_2. Cell line characteristics are shown in Supplemental Table 2. Mycoplasma testing and DNA fingerprinting were regularly performed as quality control.

High-throughput drug screening and drug libraries

Cells were seeded semi-automatically in 384-well plates (Corning) using a Multidrop dispenser (Thermo Fisher Scientific) Patient-derived cells were seeded at a concentration of 1–1.5 × 10^6 cells/ml, the seeding density of the cell lines are provided in Supplemental Table 2. On the same day, drugs were added to a final concentration of 10 nM, 100 nM or 1000 nM, using the Caliper SciClone ALH3000 liquid handling robot. The following commercially available drug libraries were utilized: Prestwick Chemical library (Prestwick Chemical, France), anti-neoplastic sequoia library (Sequoia Research Products, United Kingdom), Epigenetics library (Enzo Life Sciences, Belgium), Epigenetics screening library (Cayman Chemical, MI, USA), Spectrum collection (Microsource, CT, USA), the Cell cycle/DNA Damage compound library (MedChemExpress, Sweden), and some additional compounds (Supplemental Table 3). Upon drug exposure, cell viability was assessed by a 4-day thiazolyl blue tetrazolium bromide (MTT; Sigma-Aldrich) assay [13].

Compounds and viability assays

Pyrvinium pamoate was purchased from MedChemExpress. Venetoclax, navitoclax, etoposide, cytarabine and daunorubicin were purchased from Selleckchem. All compounds were dissolved in DMSO and subsequently diluted in medium when used for viability assays.

For the validation of the most interesting hits from our drug screen and for drug combination studies, expanded dose response curves were made using the Tecan D300 Digital Dispenser (Tecan, Switzerland) to dispense the drug. The drug response on the cell viability was assessed by a 4-day MTT assay as described elsewhere [13]. Briefly, after 4 days of incubation, 5μL/well of 5 mg/ml MTT was added to 40μL of drug-exposed cells for 6 h. Thereafter, the reaction was stopped by adding 40μL/well of a 10% SDS/0.01 M HCL solution. The next day, the absorbance of the cells was measured at wavelengths 570 nm and 720 nm using the microplate reader (Versamax). The data of 720 nm served as background noise and was subtracted from the 570 nm data. MTT data was normalized to DMSO control, tolerating a maximum concentration of ≤0.5% (v/v). The value of optical density for the controls were at least 0.07 to ensure high viability of the primary cells when assessing the effect of the drug.

Western blotting

Cell lysates were prepared in RIPA buffer (#89,901, Thermo Fisher Scientific) supplemented with a protease and phosphatase inhibitor cocktail (#78,440, Thermo Fisher Scientific). Lysates were quantified using the Pierce BCA Protein Assay Kit (#23,223 for reagent A and #23,224 for reagent B, Thermo Fisher Scientific). 25 µg lysate was loaded on pre-cast SDS-polyacrylamide gels (TGX, Bio-Rad) and transferred onto a nitrocellulose membrane using the Transblot Turbo Transfer System (Bio-Rad). Membranes were blocked in 5% milk (Elk, Campina). Proteins of interest were detected using primary antibodies against ß-catenin (1:1000, #9562, Cell Signaling) and GAPDH (1:2000, #2118, Cell Signaling), followed by IRDye680RD/800CW conjugated secondary antibodies (LI-COR Biosciences). Proteins were detected by scanning on the LI-COR Odyssey imaging system.

Mitochondria staining and pyrvinium localization

Mitochondria labeling was performed as described elsewhere, using MitoTracker Green FM (#M7514, Thermo Fisher Scientific) and Hoechst 33,342 staining (#H3570, Thermo Fisher Scientific) [14]. Cells were transferred to a µ-Slide 8 well glass bottom (#80,821, Ibidi) for live

cell imaging. Images were acquired with the confocal laser scanning microscopy platform TCS SP8 (Leica) with a 40x oil-immersion lens at a resolution of 512 × 512 pixels. Image processing was performed using the Fiji software [15].

Statistical analysis

Drug response data was analyzed using GraphPad Prism 7. Cell viability comparisons between *MLL*-rearranged AML and wildtype *MLL* AML cell lines were performed using the multiple *t*-test of average, where p-values of <0.05 were considered significant. Drug synergy was calculated using the SynergyFinder web application https://synergyfinder.fimm.fi/. [16] Concentrations where pyrvinium had a single agent toxicity of >50% were excluded from the synergy analysis.

Results

Drug library screens on patient-derived MLL*-rearranged AML cells*

We performed a high-throughput drug library screen using >4000 compounds on two primary AML patient samples carrying the *MLL-AF9* translocation. Each compound was screened at a final concentration of 10 nM, 100 nM and 1000 nM, and drugs were considered "hits" when cell viability in both leukemic samples was less than 50%, 40% and 30%, respectively. All potential drug hits were subsequently tested on two non-leukemic bone marrow samples. In total, five unique hits were identified at the 10 nM drug concentration, 28 unique hits at 100 nM, and 72 unique hits at 1000 nM (Fig. 1A). Most of the identified hits were anthracyclines, cyclin-dependent kinase (CDK) inhibitors, heat-shock protein 90 (HSP90) inhibitors, histone deacetylase (HDAC) inhibitors, or BCL2

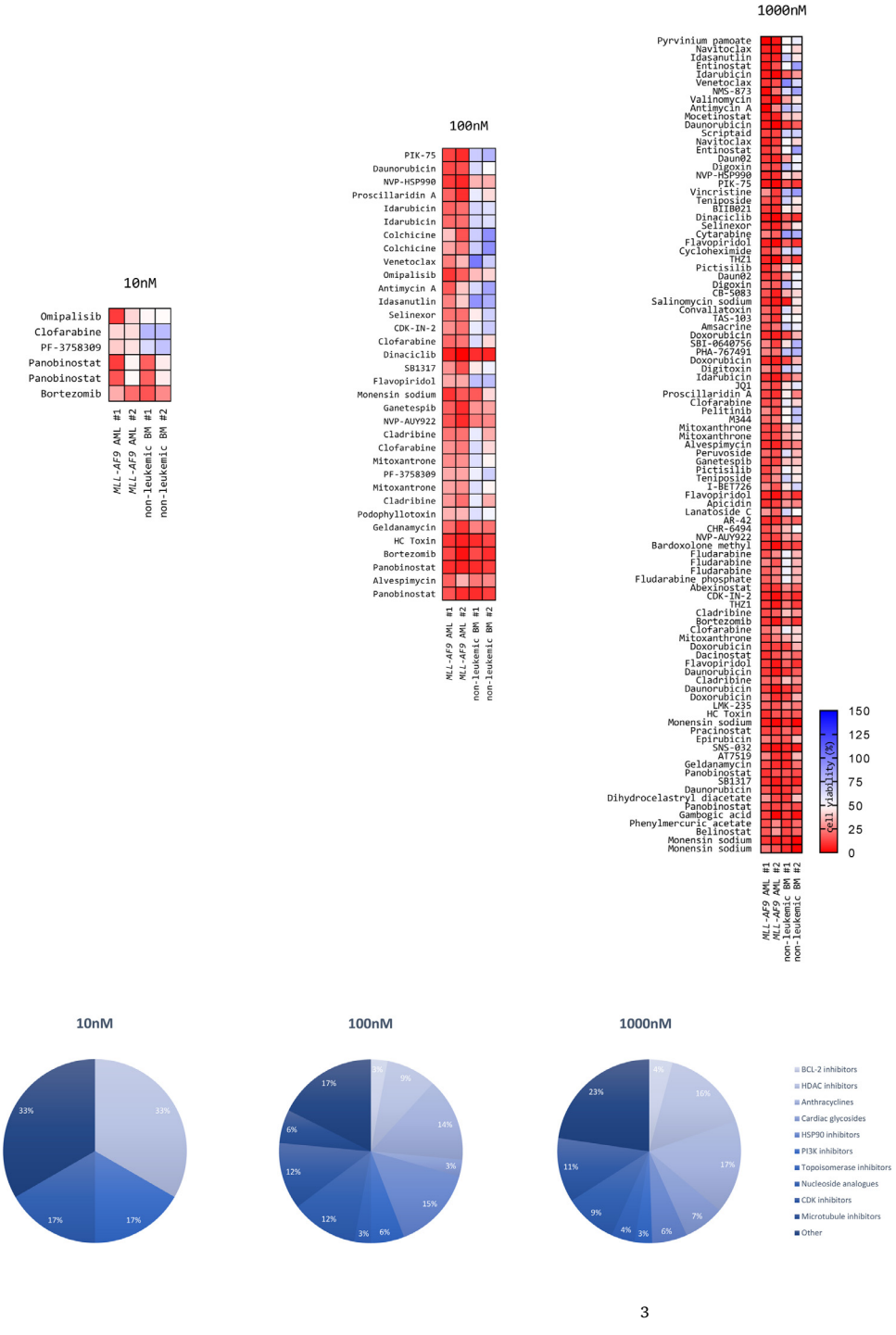

Fig. 1. *Overview of candidate drugs that were identified in the drug screening at 10 nM, 100 nM and 1000 nM on patient-derived MLL-AF9 AML cells.* (A) Heatmaps of candidate drugs identified in the drug screening at 10 nM, 100 nM and 1000 nM. Drugs are ranked based on the average cell viability of the leukemic samples *versus* the average cell viability of the non-leukemic bone marrow samples. Drug hits occurring multiple times in the heatmap indicates that the drug was present in multiple screened drug libraries. (B) Pie charts of the most occurring drug classes and targets identified in the drug screening at 10 nM, 100 nM and 1000 nM.

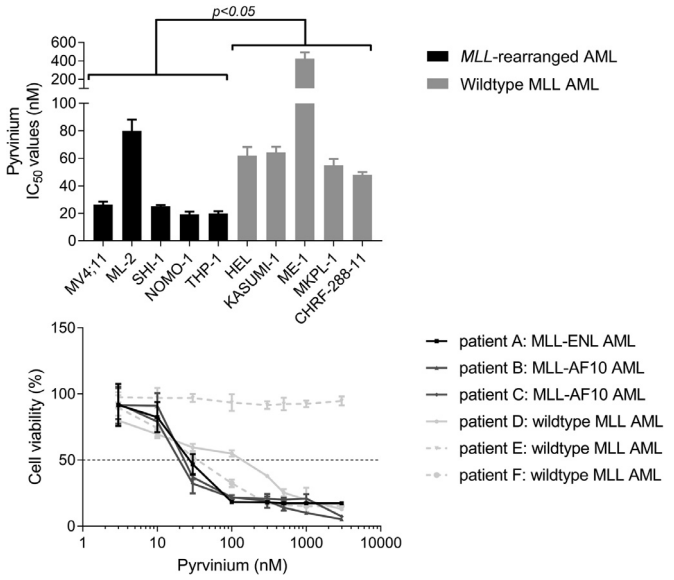

Fig. 2. *In vitro sensitivity of AML cell lines and primary AML cells to pyrvinium.* IC_{50} values for pyrvinium on five *MLL*-rearranged AML cell lines and five wildtype *MLL* AML cell lines (A). Drug responses of six primary AML samples to pyrvinium (B). Cell viability was determined by a 4-day MTT assay and data was normalized to DMSO control. Bars represent mean±standard deviation of $n = 4$ replicates.

family inhibitors (Fig. 1B; Supplemental Table 4). Anthracyclines represent established components of current treatment regimes for pediatric AML, while most of the other identified drug classes either were or are currently under clinical investigation for the treatment of AML [17–20]. Nonetheless, identifying this large amount of known drugs and similar hits throughout independent drug libraries strongly underlines the validity of our screening approach.

The hits identified at 10 nM and 100 nM have mostly been investigated and belong to the drug classes mentioned above. However, drug screening at 1000 nM identified pyrvinium as a potential novel candidate drug effective against *MLL*-rearranged AML (Fig. 1A; Supplemental Table 4). Hits were ranked using the greatest differential between anti-leukemic effect and cytotoxicity toward healthy bone marrow. Pyrvinium was the highest-ranked hit at the 1000 nM dose, eliminating virtually all of the leukemic cells, while the cell viability of non-leukemic bone marrow samples was still ~50% (Supplemental Figure 1). The effect of pyrvinium was greatest at 1000 nM, but some decrease in cell viability was already shown at the 100 nM dose. Furthermore, many CDK, HSP90 and HDAC inhibitors tested at a concentration of 1000 nM are ranked at the bottom of the heatmap due to their toxicity on non-leukemic bone marrow cells, suggesting that pyrvinium might have a therapeutic window for targeting leukemic cells.

Pyrvinium is active against MLL*-rearranged AML cell lines and primary AML cells*

To validate the efficacy of pyrvinium on additional *MLL*-rearranged AML samples, we tested pyrvinium in a full-dose response curve on $n = 5$ *MLL*-rearranged AML cell lines and on $n = 3$ *MLL*-rearranged AML patient samples (Fig. 2A,B). In addition, to identify whether pyrvinium was effective against AML cells with *MLL*-rearrangements selectively or also against AML cells with other aberrations, we included AML cells from 3 patients and 5 cell lines with wildtype *MLL*. All *MLL*-rearranged AML cell lines had an IC_{50} of ~20 nM, except for ML-2 which had an IC_{50} of 80 nM. Wildtype *MLL* cell lines had an IC_{50} of 47 nM or higher. *MLL*-rearranged AML cell lines were on average significantly more sensitive to pyrvinium than AML cell lines carrying wildtype *MLL* (Fig. 2A, Supplemental Figure S2A,B). Primary *MLL*-rearranged AML cells also

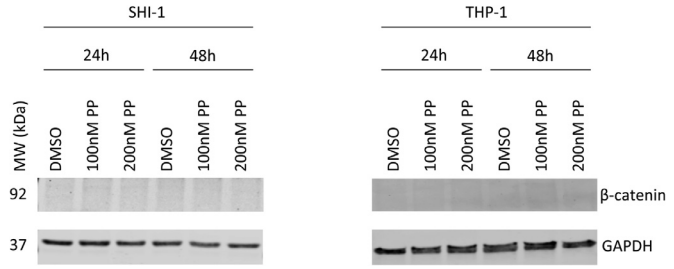

Fig. 3. *The effect of pyrvinium on β-catenin expression in MLL-rearranged AML cell lines.* β-catenin expression in SHI-1 (A) and in THP-1 (B) upon exposure to 100 nM and 200 nM pyrvinium (PP) or DMSO for 24 h and 48 h, as determined by western blot. GAPDH served as loading control.

responded well to pyrvinium, with IC_{50} values between 20 and 30 nM, including those harboring an *MLL-AF10* translocation, which is associated with a poor survival (Fig. 2B). Despite the specificity towards *MLL*-rearranged AML observed in cell line experiments, wildtype *MLL* AML patient samples D (*CBFB/MYH11*$^+$) and F (*FUS-ERG*$^+$) showed fairly similar sensitivity to pyrvinium when compared to *MLL*-rearranged patient samples. Interestingly, the patient sample E, carrying a *FLT3-ITD* mutation, was not affected at any of the pyrvinium concentrations tested (Fig. 2B).

Pyrvinium targets the mitochondria in MLL*-rearranged ALL cells*

Pyrvinium is an anthelmintic drug, though it was recently reported to possess anti-cancer properties [21]. Within hematological malignancies, studies identified that pyrvinium generally is able to inhibit tumor cell proliferation and induce apoptosis via two main described mechanisms. First, pyrvinium inhibits canonical Wnt signaling pathway by β-catenin degradation in multiple myeloma. Secondly, pyrvinium impairs mitochondrial functions in multiple hematological malignancies [14,22–25]. Here, we investigated both possible mechanisms to identify how pyrvinium exerts its anti-leukemic effects in AML cells.

To identify whether pyrvinium affects β-catenin in AML cells, two *MLL*-rearranged AML cell line models, SHI-1 and THP-1, were exposed to pyrvinium and β-catenin protein expression was assessed. However, no β-catenin protein expression was observed in either of the *MLL*-rearranged AML cell lines (Fig. 3A,B). AML cell lines carrying wildtype *MLL* showed similar results (Supplemental Figure 3A,B). The human 293T cell line was used as positive control for β-catenin protein expression (Supplemental Figure 3A). Since no β-catenin expression could be detected in our AML cell line models, we reasoned that it is unlikely that the anti-leukemic effects of pyrvinium on AML cells are mediated via inhibition of the canonical Wnt pathway.

In chronic myeloid leukemia (CML) it was shown that pyrvinium also acts in a β-catenin independent manner, and instead was found to be localized in the mitochondria where it blocked mitochondrial respiration [14]. Therefore, we performed a similar experiment on AML cell lines to investigate whether pyrvinium localizes in the mitochondria in *MLL*-rearranged AML cells. Co-localization was examined using confocal microscopy. Pyrvinium is a quinolone-derived cyanine that fluoresces red, and Mitotracker Green was used to stain the mitochondria. Our data showed that pyrvinium indeed co-localized with Mitotracker Green in both *MLL*-rearranged AML cell lines SHI-1 and THP-1 (Fig. 4A,B), indicating that the anti-leukemic effects of pyrvinium involve targeting of the mitochondria. Similar results were obtained for wildtype *MLL* AML cell lines KASUMI-1 (Supplemental Figure 4A) and HEL (Supplemental Figure 4B).

Pyrvinium in combination with conventional chemotherapy in AML

Pediatric AML is currently being treated using combination therapy, including anthracyclines (such as daunorubicin and idarubicin),

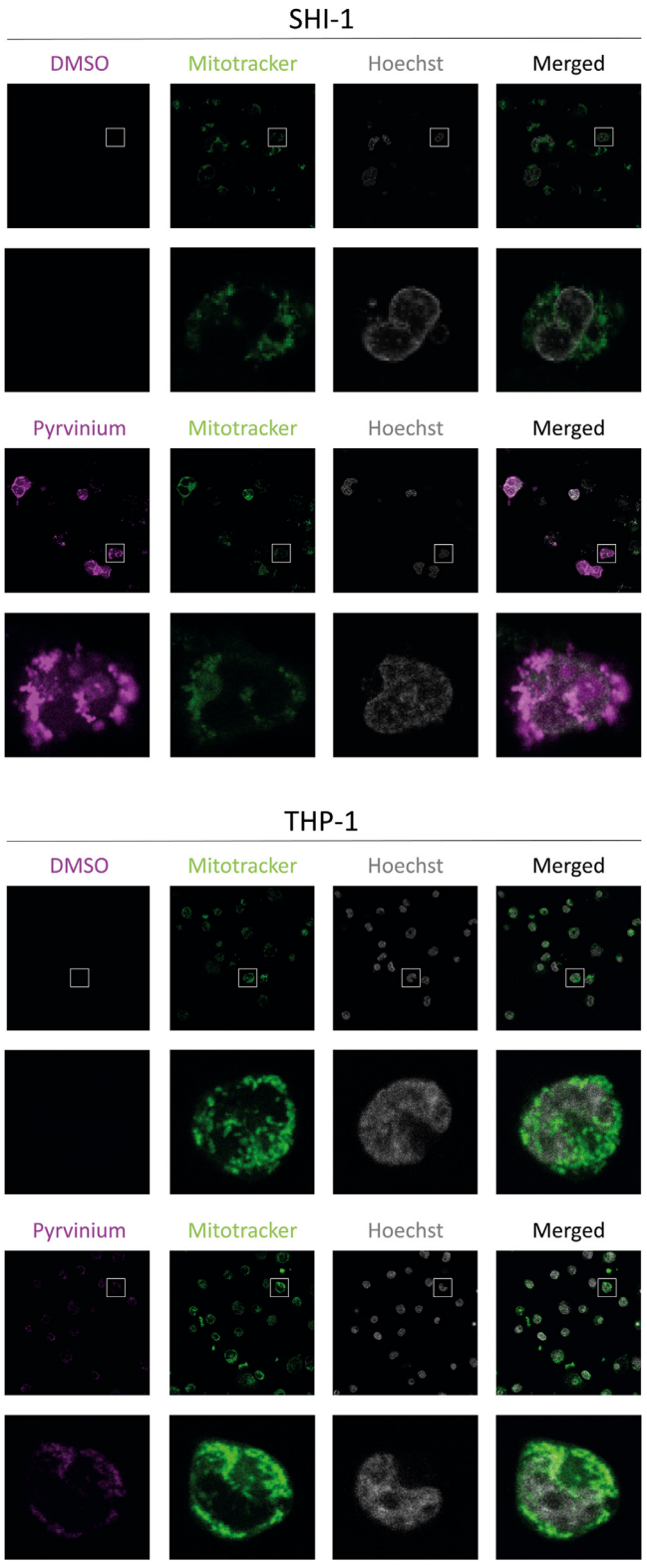

Fig. 4. *Localization of pyrvinium in the mitochondria in MLL-rearranged AML cells.* Confocal images of live cells of the SHI-1 cell line (A) and the THP-1 cell line (B) co-incubated with DMSO or 200 nM pyrvinium (magenta), the mitochondrial stain MitoTracker Green (green) and Hoechst (gray) a nuclei staining.

cytarabine, and etoposide or thioguanine. To determine whether the addition of pyrvinium does not adversely affect the efficacy of conventional chemotherapeutics, we tested the anti-leukemic effects of pyrvinium in combination with cytarabine, daunorubicin and etoposide on *MLL*-rearranged AML cell lines SHI-1 and THP-1. Although pyrvinium did not show any synergistic effects in combination with cytarabine, daunorubicin, or etoposide (Supplemental Table 5), pyrvinium did not act antagonistic either (Fig. 5A,B) and all effects in combination with conventional chemotherapeutics were additive. Similar results were obtained for AML cell lines carrying wildtype *MLL* (Supplemental Figure S5A,B; Supplemental Table 5). Overall, our *in vitro* data supports that pyrvinium might be safely implemented into current therapeutic regimens to treat patients with pediatric AML.

Discussion

To date, *MLL*-rearranged AML remains a subtype of leukemia associated with a poor outcome. To identify novel or additional therapeutic agents effective against *MLL*-rearranged AML that could rapidly be implemented into the clinic, we performed a drug library screen on primary pediatric *MLL*-rearranged AML samples.

Of note is that culturing primary acute leukemia cells *in vitro* generally comes with certain limitations, and *MLL*-rearranged AML cells are no exception. For instance, under standard culturing conditions, primary acute leukemia cells typically fail to proliferate. Therefore, using non-proliferating cells in our drug library screen, inevitably means there is a potential risk of partially underestimating the full magnitude identified drug 'hits'. This would especially be true (chemo-)therapeutics that typically rely on cell proliferation in order to exert their anti-cancer effects. Moreover, we may have missed several drug 'hits', especially in case of epigenetic-based compounds, that would have required exposure periods of more than 4 days in order reveal their anti-leukemic actions. For example, LSD1 inhibitors have shown to be effective against *MLL*-rearranged acute leukemia, but the *in vitro* anti-leukemic effects in *MLL*-rearranged AML cell line models only become apparent at day 6 of drug exposure and beyond [26]. Unfortunately, the use of primary acute leukemia cells *in vitro* culturing systems does not allow reliable drug testing over periods longer than 4 days, as shortly after these cells typically become subjected to spontaneous apoptosis. Nonetheless, we did identify numerous potential drug 'hits' (including pyrvinium) of which a substantial amount of agents had already been proven effective against *MLL*-rearranged AML (among which several anthracyclines currently used in the treatment of AML), confirming the validity of our drug screens. Apart from drugs already known to be active against *MLL*-rearranged AML, we identified pyrvinium as a novel and potentially interesting agent specifically and effectively targeting the mitochondria in *MLL*-rearranged AML cells.

As a single agent, pyrvinium effectively eliminates *MLL*-rearranged AML cells at a concentration of 1000 nM, while non-leukemic bone marrow samples were much less affected at this concentration. The latter is in agreement with previous observations. Pyrvinium was shown to induce apoptosis much more effectively in $CD34^+$ cells derived from patients with CML compared to healthy $CD34^+$ cells derived from cord blood, suggesting a degree of selectivity towards leukemic cells [14]. In addition, *MLL*-rearranged AML cells seemed more sensitive to pyrvinium than AML cells carrying the wildtype *MLL* gene, however the number of samples tested remain insufficient to draw definite conclusions regarding subtype specificity. Although this specificity would be interesting to further investigate, the aim of this study is to find novel treatment options for patients carrying *MLL*-rearranged AML as they are associated with a poor outcome.

Previous studies have described two mechanisms by which pyrvinium exerts its anti-neoplastic effects. Pyrvinium is able to inhibit the canonical Wnt signaling pathway via β-catenin degradation, but can also impair mitochondrial respiration [14,22–25]. As our data showed a lack of β-catenin expression in *MLL*-rearranged AML cells, it is unlikely

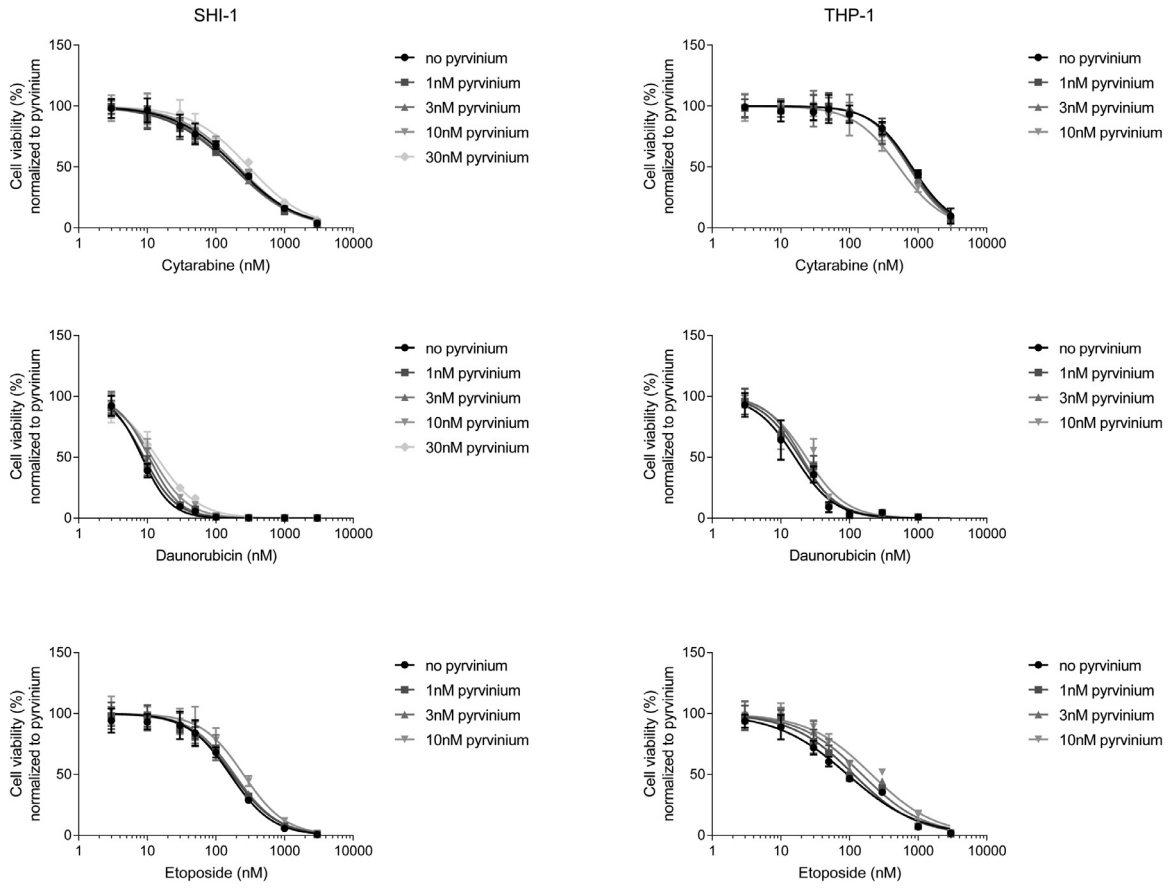

Fig. 5. *Pyrvinium in combination with conventional chemotherapeutic drugs in MLL-rearranged AML cell lines. In vitro* dose response curves (4-day MTT assays) of increasing concentrations pyrvinium in combination with increasing concentrations of cytarabine, daunorubicin or etoposide in SHI-1 (A) and THP-1 (B). Bars represent mean±standard deviation of $n = 4$ replicates. All cell viability data have been normalized for the effect of pyrvinium as single agent.

that pyrvinium affected Wnt signaling in these cells. In consistency with our data, Zhao et al. showed that genetic deletion of β-catenin did not affect the AML onset in *MLL-AF9* AML *in vivo* [27]. Instead, we demonstrate that pyrvinium is localized in the mitochondria in *MLL*-rearranged AML cells, suggesting that pyrvinium might impair mitochondrial respiration as recently shown for CML by Xiang et al. and for acute lymphoblastic leukemia (ALL) by Nair et al. [14,25]

Pyrvinium, commercially known as Povan or Vanquin, is an FDA-approved anthelmintic drug originally used for the treatment of pinworms in humans [28]. Interestingly, pre-clinical data showed that pyrvinium appeared to possess anti-cancer activity in diverse neoplastic tissues, including breast cancer, multiple myeloma, chronic myeloid leukemia and ALL [14,22,25,29].

Pyrvinium is typically administered orally, and is not absorbed from the gastrointestinal tract, hence this route of administration does not provide systemic exposure [30]. Therefore, a parenteral formulation of pyrvinium is required in case pyrvinium is to be used as a systemic anti-cancer agent. This may of course also influence its toxicity profile. Nonetheless, the observed selectivity towards leukemic cells over healthy hematopoietic cells may benefit the toxicity profile. In addition, pyrvinium analogs have recently been developed and are currently being further optimized [31]. These pyrvinium analogs might provide a better solution to the poor bioavailability. Another recent study aimed to increase targeting of pyrvinium in the bone tumor microenvironment niche by encapsulating pyrvinium in a nanoparticle delivery system and demonstrated that the system retained its anti-leukemic activity [25]. This system still needs to be tested in an *in vivo* setting, but could also offer a solution to the poor bioavailability of pyrvinium. Despite multiple reports on the anti-cancer properties of pyrvinium, this drug has not yet been investigated in clinical trials against cancer, probably due to the bioavailability. However there is precedence for use of an anthelmintic agents in a cancer setting. Mebendazole, another anthelmintic drug which also showed pre-clinical efficacy in diverse human cancers, is currently being evaluated in clinical trials against pediatric brain tumors and already has a demonstrated safety profile in a Phase I trial for adults with brain tumors (NCT02644291). Like other anthelmintic drugs, pyrvinium might also be safely repurposed as an anti-cancer therapeutic and may be an attractive drug to be tested in clinical trials for the treatment of various cancer types [21], including pediatric *MLL*-rearranged AML.

Pediatric AML is being treated using combination chemotherapy that includes anthracyclines, cytarabine and etoposide. Here we show that combinations of pyrvinium with either of these traditional chemotherapeutics all act in an additive manner. This suggests that adding pyrvinium to current treatment regimes should not negatively affect the efficacy of the established chemotherapeutic agents. In support of this, the combined use of pyrvinium with the anthracycline doxorubicin, has already proven to enhance *in vivo* efficacy in diverse xenograft mouse models of prostate cancer [32].

Conclusion

Taken together, this study demonstrates that pyrvinium is highly effective against *MLL*-rearranged AML *in vitro*, both as a single agent, as well as in combination with conventional chemotherapeutics currently used in the treatment of *MLL*-rearranged AML patients. Given its remarkable specificity towards leukemic cells, we conclude that pyrvinium is

an attractive candidate drug for further studies on aggressive types of leukemia such as *MLL*-rearranged AML.

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Declaration of Competing Interest

None.

CRediT authorship contribution statement

Priscilla Wander: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Investigation. **Susan T.C.J.M. Arentsen-Peters:** Conceptualization, Formal analysis, Investigation, Visualization, Writing - review & editing. **Sandra S. Pinhanços:** Conceptualization, Investigation, Visualization, Writing - review & editing. **Bianca Koopmans:** Methodology, Investigation, Writing - review & editing. **M.Emmy M. Dolman:** Methodology, Investigation, Writing - review & editing. **Rijndert Ariese:** Formal analysis, Writing - review & editing. **Frank L. Bos:** Formal analysis, Writing - review & editing. **Patricia Garrido Castro:** Conceptualization, Writing - review & editing, Visualization, Supervision, Funding acquisition. **Luke Jones:** Conceptualization, Writing - review & editing, Supervision. **Pauline Schneider:** Investigation, Writing - review & editing. **Miriam Guillen Navarro:** Conceptualization, Writing - review & editing. **Jan J. Molenaar:** Resources, Writing - review & editing. **Anne C. Rios:** Resources, Writing - review & editing. **C. Michel Zwaan:** Writing - review & editing, Supervision. **Ronald W. Stam:** Conceptualization, Resources, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

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Supplementary materials

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