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Pyrvinium pamoate use in a B-cell acute lymphoblastic leukemia model of the bone tumor microenvironment

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Abstract

Purpose—Pyrvinium pamoate (PP) is an anthelmintic drug that has been found to have anti-cancer activity in several cancer types. In the present study, we evaluated PP for potential anti-leukemic activity in B-cell acute lymphoblastic leukemia (ALL) cell lines, in an effort to evaluate the repurposing potential of this drug in leukemia.

Methods—ALL cells were treated with PP at various concentrations to determine its effect on cell proliferation. Metabolic function was tested by evaluating Extracellular Acidification Rate (ECAR) and Oxygen Consumption Rate (OCR). Lastly, 3D spheroids were grown, and PP was reformulated into nanoparticles to evaluate distribution effectiveness.

Results—PP was found to inhibit ALL proliferation, with varied selectivity to different ALL cell subtypes. We also found that PP's cell death activity was specific for leukemic cells, as primary normal immune cells were resistant to PP-mediated cell death. Metabolic studies indicated that PP, in part, inhibits mitochondrial oxidative phosphorylation. To increase the targeting of PP to a hypoxic bone tumor microenvironment (BTME) niche, we successfully encapsulated PP in a

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nanoparticle drug delivery system and demonstrated that it retained its anti-leukemic activity in a hemisphere assay.

Conclusion—We have demonstrated that PP is a novel therapeutic lead compound that counteracts the respiratory reprogramming found in refractory ALL cells and can be effectively formulated into a nanoparticle delivery system to target the BTME.

Keywords

B-cell acute lymphoblastic leukemia; mitochondrial respiration; drug resistance; nanoparticles

INTRODUCTION

B-cell acute lymphoblastic leukemia (ALL) originates from differentiation arrest followed by uncontrolled proliferation of the immature B lymphoid cells, resulting in accumulation within the bone marrow (BM) (1). This rapid accumulation of the leukemic blast cells leads to the “hijacking” of the BM niche and the impairment of steady state hematopoiesis. Thus, coincident with progression of disease, there is a collapse of the functional integrity of the hematopoietic system (2). The drivers of the disease have not yet been fully elucidated, but are known to include changes in chromosomal number (hyperdiploidy, hypodiploidy, trisomy 4 and 10 and intrachromosomal amplification of chromosome 21) or chromosomal translocation (MLL-AF4, ETV6-RUNX1, E2A-PBX1 and BCR-ABL1) (3). Fortunately, irrespective of the genesis of the disease, there has been continuous improvement in prognosis in both children and adults, with most achieving complete remission following treatment using the present standard-of-care therapies (4). However, disease relapse does occur in specific patients, and often the emerging disease will manifest a drug resistant phenotype, thus drastically reducing the chances of complete disease remission and cure in these patients (5, 6). One of the single most important prognostic indicators for refractory/relapsed disease is the presence of minimal residual disease (MRD) within the BM after completion of the therapeutic regimen (7, 8). Naturally, identification of therapeutic regimens that specifically target the refractory cells would lead to better therapeutic outcomes for patients who have undergone remission followed by relapse due to incomplete eradication of the MRD from the bone marrow.

In order to delineate the characteristics of the leukemic cells that constitute MRD, we previously developed a model in which ALL cells are grown in co-culture with primary human BM stromal cells (BMSC) or osteoblast cells (9). Under these conditions, the ALL cells segregate into three populations, including the cells floating in suspension (S) above the stromal cell layer, the cells loosely adhered to the top of the stromal cells, and the cells that bury underneath the stromal cells, which appear phase dim (PD) by phase contrast microscopy. Specifically, we demonstrated that the PD cells within the co-culture were more refractory to the present standard-of-care therapies when compared to S cells within the same co-culture (10). Interestingly, the drug resistant phenotype in PD cells was accompanied by a reduced proliferation index and increased expression of p27 (CDKN1B) resulting in the accumulation of these cells in the G0/G1 phase of the cell cycle. Furthermore, PD cells had an altered metabolic profile with increased expression of hexokinase 1 and 2, correlated with heightened glycolytic activity (10). Additional studies,

focused on identifying the mechanisms by which BM microenvironment-derived cues modulate resistance in PD tumor cells, demonstrated that down-regulation of miR-221/222 and BCL6 were two contributing factors important for quiescence and cell survival in the BM (11, 12). These studies highlighted the importance of understanding the mechanisms involved in the BM microenvironment-mediated protection of leukemic cells leading to MRD in ALL.

In the present study, we evaluated pyruvium pamoate (PP) as a treatment strategy for ALL, to repurpose this existing drug by potentially targeting the mitochondrial function in ALL cells, since previous studies with PP have shown promise with other types of cancers (13, 14). Our approach to evaluate PP in ALL was further underpinned by an unbiased gene expression profiling of the leukemic PD cells in co-culture with BMSC, and the observation that their mitochondrial function was altered compared to leukemic cells growing in monoculture (M). We have further characterized this functional alteration, and hypothesized that mitochondrial bioenergetics offer an actionable target for anti-leukemic therapy, specifically to target cells which are in anaerobic or hypoxic conditions (13). Although cancer cells, including leukemic cells, are highly reliant on glycolysis, several studies have indicated that targeting the mitochondrial OXPHOS as a novel treatment paradigm (15, 16). In this study, we used PP, which targets mitochondrial respiration (17), to test this hypothesis that a mitochondrial OXPHOS targeting agent will provide anti-leukemic activity *in vitro*. The choice of PP is based on its demonstrated anti-leukemic activity in nine different human-derived ALL cell lines of varied phenotypes. Furthermore, this anti-leukemic activity was, in part, associated with targeting and disruption of leukemic mitochondrial respiration. Based on these data, we suggest that agents, like PP, that target mitochondrial respiration show promise as anti-leukemic therapies. As a novel lead compound, PP may be useful in combination with the present standard-of-care to treat relapsed and refractory ALL.

MATERIALS AND METHODS

Materials and cell culture

TOM1 (DSMZ ACC#578), SUPB15 (ATCC #CRL-1929), and JM1 (ATCC #CRL-10423) were purchased and maintained in RPMI 1640 supplemented with 10 % FBS, 0.05 mM β -mercaptoethanol and 1x streptomycin/penicillin antibiotics. REH (ATCC #CRL-8286), NALM1 (ATCC #CRL-1567), NALM6 (DSMZ ACC #128), BV173 (DSMZ ACC#20), RS4 (ATCC #CRL-1873) and SD1 (DSMZ ACC#366) were purchased and maintained in RPMI 1640 supplemented with 10 % FBS and 1x streptomycin/penicillin antibiotics. Primary CD3+ T cells, peripheral blood mononuclear cells (PBMC) and bone marrow mononuclear cells (BMMC) were purchased from AllCells ([Allcells.com](https://www.allcells.com)) and maintained in Lymphocyte Growth Medium-3 (Lonza, Cat No: CC-3211) containing 10% FBS and 1x streptomycin/penicillin. Human osteoblasts (HOB) were purchased from PromoCell (Cat No: C-12720, Heidelberg, Germany) and cultured according to the vendor's recommendations. De-identified primary BMSC were provided by the WVU Cancer Institute Biospecimen Processing Core and the WVU Department of Pathology Tissue Bank. ALL cell lines were authenticated by short tandem repeat (STR) analysis (University of Arizona Genetics Core,

Tucson, AZ) and maintained in 6 % CO₂ in normoxia at 37 °C. Pyrvinium pamoate (PP) was purchased from Sigma Aldrich (Cat #P-0027) and stored at –80 °C as a 10 mM stock.

Co-culture and isolation of leukemic cell populations

Co-culture conditions were followed as previously described (9). Briefly, 1 million ALL cells were seeded on an 85% confluent BMSC layer and maintained in 5% O₂. The co-culture was fed every 4 days and PP was added, on the 9th day of culture. On the 12th day in culture, the ALL cells were isolated for further processing. The leukemic cell population that was in suspension and not interacting with the stromal cells was collected and designated as suspension cells (S). The leukemic cells that were buried under the BMSC were separated by size exclusion with Sephadex G-10 after vigorous washing to remove all leukemic cells adhered to the top of the BMSC (9). Buried leukemic cells were designated phase dim cells (PD), and have been previously described to be the most chemotherapy-resistant population (11, 12). As such, they are not assumed to be identical, but rather are used as a model for refractory tumor cells that are known to be clinically problematic in the treatment of ALL.

RNA sequencing

RNA was isolated using a Qiagen RNeasy mini kit and sent to the West Virginia University Genomics Core for next-generation sequencing. Libraries were prepared using polyadenylation selection with the KAPA Stranded mRNA-Seq Kit. Samples were sequenced as paired-end reads across two lanes using the Illumina HiSeq. Fastq files were first trimmed of any 3' adapters using Cutadapt and then aligned to the human reference genome GRCH38/hg38 with HISAT2 (16, 17). Rsubread was implemented for read counting (18). After read counting, all analyses were conducted within the R environment. Sample metrics were evaluated using both NOISeq (19, 20) and DESeq2 (21), though only DESeq2 was employed for differential expression analysis. The data has been deposited: SRA accession: PRJNA509768.

Electron transport chain complex activities

The activities of the electron transport chain (ETC) Complexes I, III, IV and V were measured as previously described (22). Briefly, REH cells in monoculture (M), or suspended in co-culture (S) or buried in co-culture (PD) were evaluated. ETC Complexes I, III, IV, and V (ATP synthase) activities were measured spectrophotometrically (23). Mitochondria were isolated from REH cells using BioVision Mammalian Mitochondria Isolation Kit for Tissue & Cultured Cells (Catalog #: K288), resuspended in 1 mL KME buffer each, and stored at –80 °C. Complex I activity was determined by measuring NADH oxidation at 340 nm. Complex III activity was assessed by measuring the reduction of cytochrome c at 550 nm in the presence of reduced decylubiquinone (50 µM). Complex IV activity was evaluated by measuring the oxidation of cytochrome c at 550 nm. Complex V (ATP Synthase) activity was measured using an assay coupled with pyruvate kinase, which converts ADP to ATP and produces pyruvate from phosphoenolpyruvate measured at 340 nm. Each treatment group was comprised of 8 replicate wells in a 96-well plate. A Bradford assay was used to measure protein concentration in isolated mitochondria used when performing calculations to obtain measurements expressed as nanomoles substrate converted/min/µg of protein. The mean measurement of each treatment group was compared with the mean of the control group

(DMSO only) with a One-Way ANOVA using GraphPad Prism 8 software following the exclusion of outliers as determined by ROUT (Q = 1%) outlier method.

The reduction of decylubiquinone in the lysate was used as a measure of Complex I activity, reduction of cytochrome c was used as a measure of Complex III, oxidation of reduced cytochrome c was used as a measure of Complex IV activity, and finally, ATP synthase activity was measured as oligomycin-sensitive ATPase activity through pyruvate kinase and phosphoenolpyruvate. Protein quantification was performed to normalize the activity measurement of each complex.

ATP measurement

Co-cultures were treated with 125 nM PP or vehicle control for 72 hrs, followed by isolation of the different cell populations, as described above. A luminescent ATP detection assay kit was used to determine the total levels of cellular ATP in 20,000 cells as per the manufacturer's instructions (abcam, Cat #ab113849). The luminescence was measured using a BioTek plate reader.

Mitochondrial staining assay

Leukemic cell populations were isolated from co-culture as described above. The isolated cells were then incubated with 100 nM of MitoTracker Deep Red FM (Invitrogen, Cat# M22426) for 30 min. After a single wash with PBS, the cells were fixed with 3.7% paraformaldehyde, and the MitoTracker intensity was ascertained using flow cytometry (BD LSR Fortessa).

Cell proliferation assay

Cells were plated in 96-well clear plates at a density of 50,000 cells per well. Cells were treated with PP at indicated concentrations, and the cell proliferation was tested after 72 hrs of incubation with the compounds. A cell counting kit was utilized according to the manufacturer's instructions (Dojindo Molecular Technologies Inc., Cat # CK04). Briefly, 10 μ l of the assay reagent was added to each well and incubated for 2 hrs at 37 °C, after which the plates were read on a BioTek Cynergy 5 plate reader at 450 nm absorbance. Vehicle-treated cells were used as controls.

Cellular metabolic effects

A Seahorse XFe96 Extracellular Flux Analyzer (Agilent, Santa Clara, CA) and Glycolysis Stress test were used to assess glycolytic activity in cells according to manufacturer's instructions. Cells were seeded into XFe96-well plates pre-coated with Cell-Tak (Corning, Corning NY) in the appropriate culture medium immediately prior to the beginning of all experiments. Cells were treated with DMSO vehicle or PP (250 nM) for 40 min, followed by metabolic flux analysis. Metabolic flux was assessed by quantification of the extracellular acidification rate (ECAR), a measurement of glycolytic function, in REH ALL cells.

One day prior to ECAR measurement assays, sensor cartridges were hydrated in XF calibrant (Agilent) and maintained in the XF PrepStation (Agilent) at 37°C overnight. The MitoStress test was used to measure OCR. On the day of the experiment, cells were

resuspended and plated directly into the 96-well plate and incubated with bicarbonate free low-buffered XF assay medium (Agilent) supplemented with L-glutamine for one hour at 37°C in a non-CO₂ incubator prior to beginning of the assay. On the day of the experiment, cells were resuspended and plated directly in with respiration buffer (XF base media supplemented with 2 mM L-glutamine) for one hr at 37°C in a CO₂ incubator prior to the assay. Glucose (10 mM), oligomycin (5 µM) and 2-deoxyglucose (2-DG; 100 mM) were added sequentially over the course of the assay. All ECAR data was collected and analyzed using Wave 2.3 software (Agilent Technologies). From the ECAR data that is collected, a variety of parameters can be calculated including glycolysis, glycolytic capacity, glycolytic reserve, and non-glycolytic acidification.

Cell death analysis

Cells were grown in 24-well plates at a density of 250,000 cells per well. Cells were treated with 250 nM of PP, 5 mM 2-DG, or 1 µM of rotenone for 24 hrs. Cells were stained using Annexin V-FITC according to manufacturer's instructions (ThermoFisher Scientific, Cat# 88-8005) and analyzed using flow cytometry (BD LSR Fortessa).

Nanoparticle encapsulation of PP

The nanoparticles were prepared as previously described (24). Briefly, 2 mg of PP was dissolved in 500 µl of acetone, along with 15 mg PEG-PLGA-COOH, and added dropwise into a 0.5% w/v solution of polyvinyl alcohol. The primary emulsion was formed by probe sonication (Heat Systems Ultrasonics Inc., Model W-225) for 10 minutes at an output level of 6 using a pulsed mode. The nanoparticles were obtained after evaporating acetone by stirring, over a period of 24 hrs at room temperature. The particle size of the nanoparticles was analyzed using a Nanosight NS300 (Malvern Panalytical, Malvern, UK). The nanoparticle samples were diluted prior to the measurements. The zeta potential of the blank and drug loaded nanoparticles was measured using the Malvern Zetasizer (Malvern, UK).

Hemosphere assay

SD1 cells were plated at 100,000 cells/well in a 96 well plate and allowed to form spheroids for 4 days. At the end of 4 days, the formation of spheroids was visualized and confirmed using light microscopy. The spheroids were then treated with 250 nM PP, nanoparticle-encapsulated PP, and the resulting effects on the spheroids were imaged using a Leica camera attached to a Leica DMIL LED microscope.

Statistical analysis

All the experiments were carried out as three independent experiments unless specifically noted otherwise in the legends. All the data were represented as mean ± SEM, and $p < 0.05$ was considered statistically significant. Statistical significance between two groups were carried out by using student's T-test. For comparing three or more groups, a one-way ANOVA was used to determine statistical significance followed by a post-hoc Tukey's test. Statistics were done using Prism 6 and Instat 3.10, both from GraphPad. For the gene expression analysis data, DESeq2 was used to calculate significance through implementation of the Wald test. In brief, adjusted p-values were derived from both the unadjusted p-value

and False Discovery Rate (FDR). DESeq2 uses a generalized linear model of computing significance across each sample, where a negative binomial model is fitted to the counts. The FDR in DESeq2 follows the Benamini-Hochberg procedure.

RESULTS

Pyrrvinium pamoate (PP) demonstrates anti-leukemic effects in ALL cells

We evaluated the effect of PP on ALL cell proliferation. Treatment with PP decreased cell proliferation in a concentration-dependent manner in eight ALL cell lines cultured in monoculture without microenvironment cues (BV173, JM1, NALM1, NALM6, REH, RS4, SUPB15 and TOM1) (Fig. 1a). The IC₅₀ of PP ranged from 0.17 μ M for REH cells to 1 μ M for RS4 cells (Fig. 1b). To determine the effect of PP on the cell cycle, we treated REH cells with PP and observed a significant decrease in the number of cells in the S and M phases of the cell cycle (Fig. S1). Subsequently, we wanted to evaluate the effects of PP on leukemic cells when they were co-cultured with BMSC, which models the bone-tumor microenvironment. Fig. S2 shows the experimental set-up of the monoculture (M), suspension (S), and phase dim (PD) cells in the culture systems. Treatment with PP resulted in a decrease in the total number of live REH cells, in both monoculture and co-culture (Fig. 1c). This overall decrease in the total number of live cells was accompanied by a significant decrease in cell viability (Fig. 1d). Specifically, a decrease in viability of ~55% was observed in M and S cell populations compared to a decrease of ~80% in the PD cell population.

Pyrrvinium pamoate inhibits mitochondrial respiration

To gain additional insight into the mechanism of PP-induced cell death in ALL cells, we analyzed its effect on mitochondrial activity. To characterize the resistant phenotype of the refractory ALL cells, we used our long-term co-culture model of REH ALL cells with BMSC to isolate the transcribed RNA from the phase dim (PD) population for comparison to transcribed RNA from cells in suspension (S) or in monoculture (M). Sample-to-sample distribution of transcript counts (Fig. S3a) illustrates the distinctive phenotypic shift in the REH PD cells, attributable to changes in global genomic expression and not to RNA content (Fig. S3). The PD cells differentially expressed roughly 2,600 genes compared to the M cells (Fig. S3d). Interestingly, no difference in gene expression profile was observed between the PD cells and S cells (Fig. S4a). Furthermore, hexokinase II (HK2) was found to be the most significantly over-expressed in the PD cell population in all of the four independent co-cultures that were used for the gene expression analysis (Fig. S4b), which corroborates our previous findings for the resistant phenotype of the REH PD cells (10). Since HK2 is an important player in glycolysis and cellular metabolism, we utilized the Ingenuity Pathway Analysis (IPA) software to explore changes in other metabolic relevant proteins of interest to us, as well as to evaluate the mitochondrial respiratory pathways. The oxidative phosphorylation module was the most significantly down-regulated pathway in the PD cells (Fig. 2a). Also, looking specifically at the mitochondrial oxidative phosphorylation pathway revealed that expression of genes in Complex I, Complex III, Complex IV, and Complex V of the electron transport chain (ETC) were down-regulated in the PD cells, while Complex II remained transcriptionally unaffected (Fig. 2b).

To validate the observations derived from the gene expression analysis, we assayed the enzymatic activity of the mitochondrial complexes in ALL cells using cell lysates from M, S and PD cells. Complex I activity of the S and PD cells was significantly reduced when compared to M cells for the REH ALL cell line (Fig. 3a). Specifically, Complex I activity was decreased by 58% in S cells and by 55% in the PD cells as compared to M cells. Complex III (Fig. 3b), IV (Fig. 3c) and V (Fig. 3d) showed no significant differences in activity with REH cells.

To gain insight into oxidative phosphorylation (OXPHOS) of the ALL cells, and how the decreased ETC complex activity of the leukemic cells in co-culture affects the mitochondrial bioenergetics, we evaluated ATP production and the relative mitochondrial activity within the different sub-populations of leukemic cells in co-culture. We found that the ATP production was significantly decreased in REH cells in co-culture with BMSC when compared to the monoculture cells (Fig. 4a). The decrease in ATP production was greater in the REH PD cells (~42%) than the S cells (~19%) when compared to the REH M cells. Furthermore, MitoTracker DeepRed fluorescence intensity, which was used as marker for mitochondrial activity, was significantly lower in the resistant PD cells when compared to the S or M cells (Fig. 4b). This decrease in mitochondrial staining was very specific and similar in PD cells, suggesting a decrease in the number of active mitochondria within this PD cell population, as compared to the monoculture (M) and co-cultured suspension (S) cells.

Treatment with 250 nM PP for 24 hrs significantly reduced the ATP production in REH (70%) cells compared to vehicle treated controls (Fig. 5a). Based on the RNA analysis, we also evaluated the effect of the addition of sodium pyruvate, which has been shown to facilitate glycolysis in cells that have dysfunctional mitochondria (25). To determine if the PP-induced death in leukemic cells could be diminished by addition of sodium pyruvate, we grew the leukemic cells in glucose-free media and supplemented the media with 10 mM sodium pyruvate. Compared to PP-treated cells growing in glucose-free media, PP-mediated cell death was significantly decreased in the cells supplemented with sodium pyruvate (Fig. 5b). Further, to ascertain whether PP-induced death in ALL cells was solely dependent on inhibition of mitochondrial respiration, we pretreated REH cells with 1 μ M rotenone, an inhibitor of Complex I of the ETC (26), as well as 2-deoxyglucose (2-DG) which inhibits glucose metabolism. Pre-treatment with rotenone was followed by treatment with 250 nM of PP. Pre-treatment of rotenone caused ~20% cell death in REH cells and treatment with PP ~10% cell death in REH (Fig. 5c). The combination of rotenone and PP however, did not lead to a significant additive or synergistic effect in cell death. In the REH cells, 2-DG did not cause a significant increase cell death, and the combination of 2-DG with PP also did not show any significant increase in cell death compared between 2DG + PP and PP-alone treated groups (Fig. 5c). Furthermore, these effects on glycolysis were supported in the mitochondrial whole cell respiration with a Seahorse Bioanalyzer (Fig. 6a), with the glycolysis stress test (Fig. 6b) indicating that PP treatment increased the glycolytic capacity of the PD cells, as shown by increased extracellular acidification (ECAR). Furthermore, PP treatment decreased the oxygen consumption in cells (OCR; Fig. 6c), corroborating findings in other cancers treated with PP.

Spheroid inhibition by pyrvinium pamoate via nanoparticle formulation

The bone tumor microenvironment (BTME) has unique metabolic demands, including a propensity for hypoxic-adapted metabolism (27). To validate the anti-leukemic activity of PP in an organoid model, we evaluated its effect in a 3D spheroid system. SD1 ALL cells were used in this assay, as unlike REH, SD1 cells are able to form 3D spheroids. We encapsulated PP within nanoparticles composed of PGLA and PEG complexes to ensure optimal penetration into the core of the spheroid due to their size <200 nm (Fig. S5) (28). We first determined if the nanoparticles (NP) by themselves had any activity on the bone marrow stromal components or the ALL cells. As shown in Fig. 7a, NP at doses as high as 100 μ M failed to elicit any death in BMSC, human osteoblasts (HOB), NALM27 ALL cells or REH ALL cells. Next, we tested the toxicity of the PP encapsulated in NP in normal cells compared to ALL cells. Fig 7B shows that 250 nM of the NP-encapsulated PP did not have any deleterious effects on BMSC, normal bone marrow mononuclear cells (BMMC), or normal peripheral blood mononuclear cells (PBMC). However, treatment with the PP NPs did cause a significant decrease (46%) in the number of live SD1 ALL cells (Fig. 7b). To ascertain that the differential effects of PP between the normal and the leukemic cells was not due to differences in cell proliferation rate, we utilized normal primary CD3+ cells and induced proliferation using a CD3/CD28 T cell activator (Fig. S6a). Treatment of dormant or proliferating T cells with PP did not result in decreased cell viability in the dormant T cell population, and showed a minimal decrease in viability (~13%) in the proliferating T cells when treated with 300 nM of PP (Fig. S6b). Finally, we wanted to determine the anti-leukemic effects of the PP-encapsulated NP in a hemosphere assay. We utilized SD1 cells to form hemospheres for 4 days, after which they were treated with 250 nM of the PP-encapsulated NP. After 48 hrs of treatment, the NP were able to penetrate the pre-formed spheres as observed from a decrease in number of hemospheres (Fig. 7c). Quantitation of the spheres using light microscopy showed that the PP-encapsulated NP resulted in a loss of existing spheres, and prevented the formation of new spheres (Fig 7d).

DISCUSSION

In the present study, we evaluated the anthelmintic drug, pyrvinium pamoate, in B-cell ALL, to evaluate the possible use of targeting mitochondrial function in this patient population. PP has been shown by others to have anti-cancer activity, but has not yet been tested in B-cell ALL. We have utilized an *in vitro* BTME co-culture system to characterize PD drug-resistant leukemic cells that mimic the cell population that constitutes minimal residual disease found within the BM of relapsed ALL patients. Furthermore, we show that the gene expression profile of proteins involved in the mitochondrial ETC complexes are downregulated in leukemia PD cells, with a significant reduction in Complex I activity, and that altered mitochondrial function was accompanied by a reduction in mitochondrial activity and reduced ATP production in PD cells. Since these PD cells are adapted for a hypoxic/anaerobic environment, we hypothesized that PP would be an effective treatment adjuvant to standard of care by targeting the altered metabolism of specifically the PD cells via inhibition of mitochondrial oxidative phosphorylation via Complex I, and thereby reduction of ATP (13). Here we show that PP inhibits mitochondrial bioenergetics and induces cell death in a variety of ALL cell lines, and importantly, is also able to specifically

target the resistant PD cells for death. The complete mechanism of PP-mediated leukemic cell death includes its ability to inhibit ATP production in addition to its mitochondrial inhibitory activity (13, 14, 29, 30). Taken together, this report demonstrates the potential for the use of PP as an anti-leukemic agent, especially in treatment of patients with relapsed refractory disease.

We had previously reported that the drug resistant ALL cell sub-population designated as the PD cells have increased glycolytic programming (10). This finding has been further validated by studies showing that B-lymphoid transcription factors (PAX5 and IKZF1) that are critical in normal B-cell development are metabolic gatekeepers that negatively regulate energy metabolism and are mutated or deleted in the majority of ALL patients (31, 32).

Furthermore, tumors from B cell ALL patients, but not T cell ALL, have fewer mitochondria than from patients with myeloid lineage leukemia, and abnormalities/irregularities in the ALL patient's mitochondria had a direct correlation to poor treatment prognosis (33). In addition, patient ALL CD34+ cells show upregulation of genes that facilitate glycolysis, and downregulation of genes related to the tricarboxylic cycle (34). In the present study, we show that our drug-resistant PD population has the highest induction in HK2, the master regulator of glycolysis (35). However, we have shown that this comes at the cost of decreased ability of the drug-resistant ALL cells to utilize oxidative phosphorylation for their energy needs. Specifically, we have demonstrated that the activity of mitochondrial Complex I was severely decreased in ALL cells in co-culture with BMSC, and this was accompanied by decreased ATP production and mitochondrial activity within the drug resistant PD cells.

Sensitization of ALL cells to chemotherapy has been achieved recently by the use of an antibiotic, tigecycline, that acts through inhibition of mitochondrial respiration (36). This finding supported our evaluation of PP in leukemia, targeting a mitochondrial component in cancers. In our study, we selected PP as our agent of choice for inhibiting mitochondrial respiration in ALL cells. PP has been shown to have activity in both solid tumors, like breast cancer and prostate cancer (37, 38), and hematological malignancies, like chronic myeloid leukemia and multiple myeloma (13, 30); however, its activity in ALL has not yet been reported. PP exerts its cytotoxic activity by inhibiting Complex I in mammalian cells (13); and under hypoxia, additionally inhibiting Complex III activity (29). The cytotoxic activity of PP was found to be exaggerated when cancer cells were grown in hypoglycemic conditions, such as that found within the tumor microenvironment (39). In the present study, we show that PP demonstrates anti-leukemic activity in different ALL cell lines, irrespective of their inherent mechanism of malignancy. Indeed, REH ALL cells, which are driven by the ETV6-RUNX1 oncogene, and TOM1 ALL cells driven by the BCR-ABL oncogene, were equally sensitive to PP (data not shown). Furthermore, we were able to show that this anti-leukemic effect coincided with a rapid fall in ATP levels in REH cells, which indicated that PP targeted and inhibited mitochondrial respiration. Most importantly, PP was specifically active in the drug-resistant PD cells, indicating its potential for inducing death in drug resistant cells found within the relapsed refractory patients.

Previously we had shown that the Complex I inhibitor, rotenone, induced a complete inhibition of oxygen consumption rate in ALL cells (10). In our current study, pre-treatment

of ALL cells with rotenone did not abolish the anti-leukemic activity of PP, suggesting that PP does not antagonize the Complex I inhibition of rotenone. Alternative or additional effects of PP to those observed on the mitochondria may include the inhibition of the Wnt/ β -catenin pathway, as has been suggested for inhibition in multiple myeloma (40,41,42,43,44); this is a mechanism we will explore in the future in more detail.

PP is an FDA-approved drug and is safe for human use in an oral dosage form for treatment of pinworms (15). However, its systemic bioavailability after oral administration is negligible, making it a very poor choice of agent for use in treating leukemia (45). A study looking at the *in vivo* effect of PP on androgen receptor has shown that intraperitoneal injections of 1 mg/kg results in a peak plasma levels of 150 nM, which is near the IC₅₀ value of PP used in our present study (46). Unfortunately, this dose was shown to cause toxicity as seen by a rapid weight loss in the treated mice. To overcome this disadvantage, we utilized a previously well-characterized nanoparticle delivery system made of PEG-PGLA polymers (47, 48). Using this delivery system, we show that PP can be encapsulated and delivered to tightly aggregated leukemic spheroids without losing activity. Also, the PP encapsulated nanoparticles showed specificity in their cytotoxicity by sparing normal mononuclear and stromal cells while, efficiently inducing death in ALL cells.

Even though a lot of attention has been given to targeting of glycolysis in cancer cells as a strategy for therapy, a phase I clinical study utilizing a glycolytic inhibitor, 2-deoxy-D-glucose, has been shown to be well tolerated but not very successful in reversing disease progression (49). Interestingly, inhibitors of the mitochondrial Complex I have now been successfully tested in pre-clinical studies, and are entering clinical trials for use against leukemia and lymphomas (www.clinicaltrials.gov; Identifier: and). Success of mitochondrial inhibitors has been attributed to increased reliance of tumor cells on both glycolysis and oxidative phosphorylation for their energy needs, and while inhibition of oxidative phosphorylation in normal cells can allow cells to switch to glycolysis and still provide for their energy needs, such adaptation is not possible in cancer cells where glycolysis is already upregulated (50). However, this hypothesis needs to be further tested and confirmed in an *in vivo* scenario.

CONCLUSION

In the present study, we have demonstrated that PP is a novel B-cell ALL chemotherapeutic compound, affecting ALL cells in a BTME differently than circulating cells. Furthermore, we were successfully able to formulate a nanoparticle drug delivery system that could potentially increase the systemic availability of PP in leukemic patients. In light of our reported data, PP has a promising activity that can be exploited in the development of mitochondrially-targeted strategies, in combination with standard of care, in the clinic to treat ALL patients that have relapsed, and are refractory to the existing standard-of-care.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

2-DG	2-deoxyglucose
ALL	B-cell acute lymphoblastic leukemia
ATP	adenosine triphosphate
BM	bone marrow
BMSC	bone marrow stromal cells
BTME	bone tumor microenvironment
ECAR	extracellular acidification rate
ETC	electron transport chain
FBS	fetal bovine serum
FCCP	carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazine
HK2	hexokinase II
HOB	human osteoblasts
IPA	Ingenuity Pathway Analysis
M	monoculture
MRD	minimal residual disease
NP	nanoparticles
OCR	oxygen consumption rate
OXPHOS	oxidative phosphorylation
PD	phase dim, buried co-culture ALL cells
PP	pyrvinium pamoate
TBS	Tris-buffered saline
S	co-culture ALL cells in suspension

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Fig. 1. Anti-leukemic activity of pyrvinium pamoate

Eight ALL cell lines were treated with increasing concentrations of pyrvinium pamoate for 72 hrs. Cell proliferation was measured using a cell counting kit as described in the methods (a). IC_{50} values were calculated from the dose response curve for each cell line using the CompuSyn software (ComboSyn, Inc.) (b). REH cells were grown in monoculture (M) or in co-culture with BMSC in a 24 well plate for 12 days. On day 9 in co-culture, the cells were treated with 250 nM pyrvinium pamoate (PP). The live cells were counted using the Trypan blue exclusion method for cells grown alone (M), cells in suspension (S), and cells buried (PD) when in co-culture with BMSC (c). The total number of cells and the number of live cells were used to compute the % viability (d). The data are presented as mean $\pm$ SEM and is a representative of a single experiment done in triplicate and performed three independent times. * $p < 0.05$, when compared to corresponding untreated group, ** $p < 0.05$, when compared to all other untreated and treatment groups.

Fig. 2. Metabolic changes in REH PD cells in co-culture with BMSC

The top eight altered metabolic pathways were determined by cumulative p-values within a pathway, along with the size of the pathway (orange line). The number next to each bar indicates the proteins in each pathway, while the colored legends describes the changes in expression (**a**). IPA pathway examining changes to mitochondrial complex transcripts. Green indicates significantly decreased expression in the PD cells compared to cells grown in monoculture. Grey indicates no change in gene expression (**b**). Complex I, III, IV, and V had changes in gene expression, while no changes were observed in Complex II.

Fig. 3. Electron transport chain complex activity in leukemic cells in co-culture with BMSC
REH cells were maintained in long-term co-culture with BMSC as described in the methods. Following 12 days in culture, leukemic cells growing in monoculture (M), suspension leukemic cells from co-culture (S), and phase dim leukemic cells from co-culture (PD) were collected, and the activities of Complex I (a), Complex III (b), Complex IV (c) and Complex V (d) were measured. Complex I was the only mitochondrial complex that had a change in enzymatic activity. The data are presented as mean $\pm$ SEM and represent three experiments conducted independently. * $p < 0.05$, as compared to cells grown in media (M).

Fig. 4. Characterization of mitochondrial function in ALL cells in co-culture with BMSC
 REH were maintained in long-term co-culture with BMSC as described in the methods. Following 12 days in culture, the ALL cells growing in monoculture (M), suspension cells (S), and the phase dim cells (PD) were isolated and either subjected to ATP level detection and quantitated as relative luminescence intensity (**a**) or mitochondrial staining with MitoTracker Deep Red and quantitated as mean fluorescence intensity (**b**) as described in the method section, showing a reduction in mitochondrial OXPHOS function. The data are presented as mean $\pm$ SEM and is a representative of a single experiment done in triplicate and performed three independent times. * $p < 0.05$, when compared to cells grown in monoculture (M), ** $p < 0.05$, when compared to cells grown in monoculture (M) or the cells in suspension in co-culture (S).

Fig. 5. Pyrvinium pamoate's anti-leukemic activity requires inhibition of mitochondrial respiration

REH cells were plated at 250,000 cells/well in a 24 well plate and then treated with 250 nM pyrvinium pamoate (PP) for 24 hrs. Following treatment, total ATP was measured and quantitated as the relative luminescence intensity (RLI) as described in the methods (a). REH cells were grown overnight in glucose-free media or glucose-free media supplemented with 10 mM sodium pyruvate. After overnight incubation, the cells were treated with 250 nM of PP and cell death was analyzed after 24 hrs using flow cytometry (b). REH cells were pre-treated for 2 hrs with rotenone, and followed by treatment with pyrvinium pamoate (ROT + PP) for 24 hrs. Cells treated with rotenone alone (ROT) or pyrvinium pamoate alone (PP) were used for comparison. Additionally, cells were also treated with 2-DG. Untreated cells were used as control to measure drug-mediated cell death by annexin V binding assay (c). The data are presented as mean $\pm$ SEM and is a representative of a single experiment done in triplicates and performed three independent times. * $p < 0.05$, when compared to all other untreated and treatment groups.

Fig. 6. Metabolic effects of PP treatment on ALL cells

REH cells were plated at 20,000 cells per well in a Seahorse Bioscience Flux plate.

Treatment with PP changed the extracellular acidification rate (ECAR) for both glycolysis (a) and the glycolytic capacity (b) of cells, which are two parameters that can be calculated from the ECAR measurements obtained when performing a Glycolysis Stress Test.

Treatment with PP reduced the oxygen consumption rate (OCR) in cells. Each bar represents mean $\pm$ S.D where $n = 6$.

Fig. 7. Pyrvinium pamoate demonstrates anti-leukemic activity in 3D spheroids.

BMSC, normal human osteoblasts (HOB), and ALL cell lines, NALM27 and REH, were plated at 50,000 cells/well in a 96 well plate and treated with increasing concentrations of empty nanoparticles (NP[Empty]) for 72 hrs. Cell proliferation was measured using a cell counting kit as described in the methods (a). BMSC, normal bone marrow mononuclear cells (BMMC), normal peripheral blood mononuclear cells (PBMC), and an ALL cell line (SD1) were plated at 50,000 cells/well in a 96 well plate and treated with 250 nM pyrvinium pamoate encapsulated in nanoparticles for 72 hrs. Cell proliferation was measured using a cell counting kit as described in the methods (b). Light microscope images of SD1 hemospheres that were allowed to form for 4 days, followed by treatment with 250 nM of PP, and PP encapsulated in nanoparticles (SD1 spheres + NP [PP]) for 48 hrs (c). The number of spheres were quantitated (d). Empty nanoparticles were used as a control. The data are presented as mean $\pm$ SEM and is a representative of a single experiment done in triplicate and performed three independent times. * $p < 0.05$, when compared to all other untreated and treatment groups.