Molecular mechanism of c-Myc and PRPS1/2 against thiopurine resistance in Burkitt's lymphoma

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Abstract
Patients with relapsed/refractory Burkitt's lymphoma (BL) have a dismal prognosis. Current research efforts aim to increase cure rates by identifying high-risk patients in need of more intensive or novel therapy. The 8q24 chromosomal translocation of the c-Myc gene, a main molecular marker of BL, is related to the metabolism by regulating phosphoribosyl pyrophosphate synthetase 2 (PRPS2). In our study, BL showed significant resistance to thiopurines. PRPS2 homologous isoenzyme, PRPS1, was demonstrated to play the main role in thiopurine resistance. c-Myc did not have direct effects on thiopurine resistance in BL for only driving PRPS2. PRPS1 wild type (WT) showed different resistance to 6-mercaptopurine (6-mp) in different metabolic cells because it could be inhibited by adenosine diphosphate or guanosine diphosphate negative feedback. PRPS1 A190T mutant could dramatically increase thiopurine resistance in BL. The interim analysis of the Treatment Regimen for Children or Adolescent with mature B cell non-Hodgkin's lymphoma in China (CCCG-B-NHL-2015 study) confirms the value of high-dose methotrexate (MTX) and cytarabine (ARA-C) in high-risk pediatric patients with BL. However, there remains a subgroup of patients with lactate dehydrogenase higher than four times of the normal value (4N) for whom novel treatments are needed. Notably, we found that the combination of thiopurines and the phosphoribosylglycinamide formyltransferase (GART) inhibitor lometrexol could serve as a therapeutic strategy to overcome thiopurine resistance in BL.

KEYWORDS
Burkitt's lymphoma, c-Myc, PRPS1/2, thiopurine resistance
1 | INTRODUCTION

Burkitt’s lymphoma (BL) is one of the highly invasive mature B cell malignant tumours, and its incidence rate is about 4.5/1 000 000. The genetic characteristic of BL is the 8q24 chromosomal translocation of the c-Myc gene which is the main marker gene of BL. c-Myc gene plays a crucial role in many important physiological activities such as cell division and proliferation, metabolism, cell cycle and apoptosis. It has been found that the activated c-Myc gene can induce the tumour metabolism. Therefore, the therapy of targeting c-Myc is a trend in the treatment of high-risk and relapsed or progressive BL.

Davide Ruggero demonstrated that c-Myc could regulate the metabolic changes of tumour cells by regulating the rate-limiting enzyme phosphoribosyl pyrophosphate synthetase 2 (PRPS2). PRPS1 and PRPS2 which catalyse the same biochemical reaction are critical rate-limiting purine biosynthesis enzymes. However, PRPS1 has higher enzymatic and stronger allosteric activity than PRPS2. PRPS1 can also be significantly inhibited by adenosine diphosphate (ADP) and guanosine diphosphate (GDP) negative feedback. It was reported that negative feedback-defective PRPS1 mutants could drive thiopurine resistance in relapsed childhood acute lymphoblastic leukaemia (ALL). It is unknown whether c-Myc and PRPS2 are associated with the thiopurine resistance.

In the current study, we compared the characteristic of chemotherapy drug resistance in BL with that in leukaemia and dissected the roles of c-Myc, PRPS1 and PRPS2 in thiouprine drug resistance in BL. BL showed great thiopurine resistance. PRPS1 played the key role in thiopurine resistance which showed different resistance to the thiopurines in different metabolic cells for the feedback inhibition of ADP/GDP. The interim analysis of the CCCG-B-NHL-2015 study confirms the value of high-dose (HD) MTX and ARA-C in high-risk paediatric patients with BL. However, there remains a subgroup of patients with lactate dehydrogenase (LDH) >4N for whom novel treatments are needed. The combination of thiopurine and the GART inhibitor lometrexol can increase the cell sensitivity to thiopurine resistance in BL. These results provide a new therapeutic strategy to overcome thiopurine drug resistance in BL.

2 | MATERIALS AND METHODS

2.1 | Cell culture

The Namalwa, Daudi, Raji BL cell lines and Reh, Molt4, Nalm6, Vocc6 leukaemia cell lines were cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS) (HyClone), 100 U/mL penicillin G and 100 μg/mL streptomycin. HEK-293T cells and human fibroblast (HF) cells were cultured in DMEM supplemented with 10% FBS (HyClone), 100 U/mL penicillin G and 100 μg/mL streptomycin. All cells were incubated at 37°C in 5% CO2 unless otherwise specified.

2.2 | Cell construction

Phosphoribosyl pyrophosphate synthetase 1 WT, PRPS2 WT, c-Myc coding DNA sequences were cloned into a pGV287 green fluorescent protein (GFP) vector. The PRPS1 A190T coding region was cloned into pGV303 GFP vector (Shanghai GeneChem). To produce the lentivirus, each expression vector was transfected into 293T cells with second-generation lentiviral packaging plasmids pMD2. G and psPX2 using the PolyExpression transfection reagent (Excellgen). 48 and 72 hours after transfection, we harvested the culture medium, incubated with Lenti-X concentrator (Clontech Laboratories), and centrifuged to obtain the concentrated lentivirus. The 293T cells were infected with the lentiviruses in the presence of 6 μg/mL polybrene (Sigma-Aldrich) for 24 hours. Overexpression was confirmed by Western blot.

2.3 | Western blot

Treated cells were harvested in lysis buffer and analysed by SDS-PAGE with the following antibodies: c-Myc (1:1000 dilution, Abcam, Cambridge, US), PRPS1 (1:1000 dilution, Santa, California, USA), PRPS2 (1:1000 dilution, Invitrogen, California, USA), flag-tag (1:1000 dilution, Huaan, Hangzhou, China), his-tag (1:1000 dilution, Huaan, Hangzhou, China) and actin (1:10 000 dilution, Huaan, Hangzhou, China). Immunoblots were analysed using the Odyssey system (LI-COR Biosciences, Lincoln, Nebraska, USA).

2.4 | Cell viability and apoptosis

Cell viability was determined by using the CellTiter-Glo Luminescent kit (Promega, Madison, Wisconsin, USA) according to the manufacturer’s instructions. Cells were seeded in 96-well plates (10 000 cells per well) and treated for 72 hours with serially diluted drugs. CellTiter-Glo reagents (50 μL) was added to each well and mixed for 10 minutes before the luminescence was measured on a microplate reader (BioTek, Vermont, USA). Apoptosis was measured by staining with annexin V-APC and Propidium Iodide (PI)-phycocerythrin (PE) (Annexin V-APC Apoptosis Detection kit, BD Pharmingen), followed by flow cytometry on a FACS flow cytometer (BD, Canto II). All experiments were performed in triplicate, and results were calculated as the mean ± SD.

2.5 | Stable gene knockdown

Lentiviral shRNAs were used to knock down c-Myc expression in Raji, Daudi and Vocc6 cells. pLKO.1 shRNA vector and scrambled control pLKO.1 shRNA were purchased from Thermo Fisher Scientific. The knockdown efficiency of each shRNA was tested by Western blot. Clustered regularly interspaced short palindromic repeats (CRISPRs) were designed based on information available at
FIGURE 1  Drug resistance to thiopurines related to high c-Myc expression as well as nucleotide metabolic level. A-E, Viability of cells, including Burkitt’s lymphoma cell lines (Daudi, Namalwa and Raji), ALL cell lines (Nalm6, Vocab6 and Molt4) at increasing concentrations of c-Myc inhibitor 10058-f4 (A), thiopurines (6-mp/6-TG) (B), ARA-C (C), 5-fu (D), MTX (E). F, Heatmap showing intracellular steady-state metabolite profiles of Burkitt’s lymphoma cell lines (Daudi, Namalwa and Raji), ALL cell lines (Nalm6, Vocab6 and Molt4). Red and green colours depict high and low nucleotide metabolism levels, respectively. Levels are scaled based on metabolism values that have been mean-centred to zero. G, Representative Western blot analysis of c-Myc and purine biosynthesis enzymes (PRPS1/2) in Burkitt’s lymphoma cell lines (Daudi, Namalwa and Raji), ALL cell lines (Nalm6, Vocab6 and Molt4). β-Actin was used as a loading control. ALL, acute lymphoblastic leukaemia.
**FIGURE 2** Direct manipulation of c-Myc has no effects on thiopurine drug resistance. A, Western blot analysis of oncogene c-Myc in Namalwa after treating with increasing concentrations of c-Myc inhibitor 10058-f4 (0, 10, 20, 30 µg/mL) for 48 h. β-Actin was used as a loading control. B, Apoptosis of Namalwa after treating for 48 h with increasing concentrations of c-Myc inhibitor 10058-f4 (0, 10, 20, 30 µg/mL). *: P < 0.05; two-tailed Student’s t tests. C, The 6-mpIC50 of Burkitt’s lymphoma cells including Namalwa, Daudi and Raji treated with or without 20 µg/mL c-Myc inhibitor 10058-f4 for 48 h. P > 0.05; two-tailed Student’s t tests. D, Heatmap showing metabolomics analysis of Namalwa after treating with or without 20 µg/mL c-Myc inhibitor 10058-f4. E, c-Myc and PRPS1/2 in a Western blot analysis of sh-c-Myc Raji cells. β-Actin was used as a loading control. F, Cell viability of sh-c-Myc Raji cells at increasing concentrations of 6-mp. P > 0.05; two-tailed Student’s t tests. G, Heatmap showing intracellular steady-state metabolite profiles of sh-c-Myc Raji cells. H, Western blot of c-Myc and PRPS1/2 in c-Myc overexpressed Vocb6cells. β-Actin was used as a loading control. I, The IC50 of thiopurines in c-Myc overexpressed Vocb6cells. P > 0.05; two-tailed Student’s t tests. J, Heatmap showing intracellular steady-state metabolite profiles of cell lines in (H). K, Western blot of c-Myc and PRPS1/2 in sh-c-MycVocb6cells. β-Actin was used as a loading control. L, Testing the 6-mpIC50 of Vocb6sh-c-Myc cell line. P > 0.05; two-tailed Student’s t tests. M, Heatmap showing purine metabolite profiles of cell lines in (K).

http://crispr.mit.edu and were cloned into the lentiCRISPR/Cas9 vector (Addgene, Watertown, USA) by following the Zhang laboratory’s protocol. The sequence targeted by PRPS1 CRISPR is 5′-TTGGTCCTTACCAGGTCTCC-3′, and the sequence targeted by PRPS2 CRISPR is 5′-GGATGATAGCGCAATCTTG-3′.

### 2.6 Metabolite flux

Cells were cultured for 48 hours in RPMI 1640, then harvested and pelleted. The reaction was quenched in cold 80% methanol, cells were centrifuged at 1200 g for 10 minutes, and metabolites in the supernatant were analysed by LC-MS. The relative concentrations were defined according to the standard curve of compounds dissolved in 80% methanol without correcting for cell matrix effect.

### 2.7 Reverse transcription-PCR analysis

MSH2 cDNA fragments containing the region between exon 9 and exon 16 were amplified by reverse transcription-PCR with TaKaRa RNA PCR Kit (AMV) Ver.3.0 using Nalm6 and Reh total RNA as template. The primer sets were as follows:
Forward: 5′-AAGCTGATTCGCGTGGTCTC-3′.
Reverse: 5′-TCGAAACCTCTACCTTT-3′.

### 2.8 Statistical analysis

All experiments were repeated three times. Data are presented as mean ± SD. Two-tailed Student’s t tests were performed, and P < 0.05 was considered statistically significant.

### 3 RESULTS

#### 3.1 Drug resistance to thiopurines related to high c-Myc expression as well as nucleotide metabolic level

In BL, some patients do not respond to initial therapy or relapse after the standard therapy, which leads to poor prognosis. The mechanisms underlying BL chemoresistance remain poorly defined. To uncover the intrinsic mechanism, we used several cell lines of BL (Daudi, Namalwa and Raji) and ALL (Molt4, Vocb6 and Nalm6) as model. First, we detected half inhibit concentration (IC50) of several key chemotherapy drugs, such as 6-mercaptopurine(6-mp), 6-thioguanine (6-TG), cytarabine (ARA-C), methotrexate (MTX), 5-fluorouracil (5-FU) and c-Myc inhibitor 10058-f4 in all cell lines (Figure 1A-E). We found that there was no difference on drug resistance to 5-FU, ARA-C and 10058-f4 in two disease cell lines and MTX showed more sensitive in BL than in ALL cell lines (Figure 1A, C, D, E). According to our results, the resistance to thiopurines, 6-mp and 6-TG, was significantly higher in BL cells than in ALL cell lines (Figure 1B). We then examined the level of nucleotide metabolism in all cell lines because thiopurines targeted at the purine metabolic pathway. BL cells displayed the increased levels of nucleotide metabolism including purine and pyrimidine nucleotides, especially ADP, GDP, inosine monophosphate (IMP), hypoxanthine (HX) and so on (Figure 1F).

The most important genetic characteristic of BL is the amplification of the c-Myc gene which regulates PRPS2 expression to drive tumour metabolism. We detected the protein expression of c-Myc and PRPS1/2 in different cell lines. The protein expression of c-Myc and PRPS1/2 in BL cells was significantly increased. (Figure 1G). Therefore, it suggested that the high expression of c-Myc gene led to the high level of nucleotide metabolism resulting in the thiopurine resistance in BL.

#### 3.2 Direct manipulation of c-Myc has no effects on thiopurine drug resistance

To confirm that c-Myc was related to thiopurine drug resistance, we first used three different concentrations of a small molecular c-Myc inhibitor, 10058-F4, to decrease the level of c-Myc in BL by inhibiting the c-Myc/Max heterodimerization (Figure 2A). And the high concentration of the c-Myc inhibitor could dramatically increase cell apoptosis (Figure 2B). We then analysed the effects of different concentrations of 10058-f4 in response to 6-mp in different BL cell lines. According to our data, no matter what concentration of 10058-f4 was, IC50 of 6-MP showed no significant decrease (Figure 2A). What’s more, the combination of 10058-f4 and 6-mp did not affect the thiopurine resistance in all BL cells (Figure 2C). Identical
with the data of drug sensitivity, the nucleotide metabolic level in BL cells showed no difference between cells treated with and without 10058-f4 (Figure 2D). It suggested that c-Myc may have no direct function in 6-mp drug resistance.

Then, we knocked down c-Myc in high metabolic BL cell lines (Figure 2E, Figure S2A) and low metabolic Vöcb6 ALL cell line (Figure 2K), whereas we overexpressed c-Myc in low metabolic ALL cell lines (Figure 2H, Figure S2C). The c-Myc knockdown had little effects on thiopurine drug resistance (Figure 2F, Figure 2B), and the c-Myc overexpressed cell lines conferred thiopurine resistance similarly to control cells as well (Figure 2I, Figure S2D). Then, we detected the level of nucleotide metabolism in c-Myc regulated BL and ALL cell lines mentioned above. The regulation of c-Myc also had faint influences on purine metabolism (Figure 2G,j,m, Figure S2E). Therefore, our results suggested that direct regulation of c-Myc did not affect thiopurine drug resistance. What’s more, PRPS2 may have no function on thiopurine resistance, and some other molecules regulating the purine metabolism remain uncovered.

3.3 | PRPS1 plays the key role in the thiopurine resistance regulated by the level of purine metabolites such as IMP, ADP and GDP

On the basis of the role of PRPS1 and PRPS2 in purine biosynthesis, we examined whether the regulation of PRPS1 or PRPS2 had the same thiopurine drug resistance in different metabolic cell lines. We established ALL and BL cell lines infected with PRPS1 wild type (1-wt), PRPS2 wild type (2-wt), PRPS1 knocked out (1-ko) and PRPS2 knocked out (2-ko) retroviruses (Figure 3A,D, Figure S3A,C,E). In low purine metabolic ALL cell lines, the overexpression of PRPS1 obviously increased the level of thiopurine drug resistance, while overexpressed PRPS2 did not obviously change the 6-mpIC50 (Figure 3B, Figure S3B). Moreover, knocking out PRPS1 in BL and ALL cell lines could dramatically decrease the 6-mp drug resistance (Figure 3B,E, Figure S3B,D,F). We observed that Vöcb6PRPS1-WT cell line had higher purine metabolism than Vöcb6PRPS2-WT cell line as well (Figure 3C). As shown in Figure 3E, Figure S3D,F, overexpression of PRPS1 or PRPS2 in high metabolic cell lines showed little effects on thiopurine drug resistance. Moreover, similar to the ALL cell lines, knocking out PRPS1 in BL cell line could significantly decrease the IC50 of 6-mp. Knockout of PRPS2 only slightly decreased the IC50 of 6-mp (Figure 3E, Figure S3D,F). In addition, we investigated that PRPS2 WT showed no difference in purine metabolism with PRPS1 WT Namalwa cells, consistent with 6-mp resistance data mentioned above (Figure 3F). The results suggested that PRPS1 played the main role in the thiopurine resistance, while PRPS2 did not affect the drug sensitivity significantly.

Nalm6 is a MSH2-deficient precursor B cell lymphoblastic leukaemia (BCP-ALL) cell line. To know whether the PRPS1 A190T mutation in BL and ALL cell lines to select appropriate concentrations (Figure S1B) and then tested the thiopurine resistance after adding HX or ADP. In all cancer cells, adding HX highly increased the resistance to thiopurine drugs in the same level (Figure 3G). Therefore, in BL, HX was significantly higher than that in ALL cell lines, which was one reason for drug resistance to 6-mp (Figure 1F). Adding ADP significantly led to higher sensitivity of thiopurine drugs in all PRPS1 overexpressed cells, while PRPS2 overexpressing was resistant to the effects of ADP (Figure 3H). In BL, ADP and GDP were also significantly higher than those in ALL (Figure 1F). Therefore, overexpression of PRPS1 could not exhibit the enzymatic activity or affect the IC50 of 6-mp. Our results suggested that high HX and ADP/GDP manipulated PRPS1 activity and the 6-mp IC50. As a result, it implicates that PRPS1, not PRPS2, is the key factor in thiopurine resistance.

3.4 | PRPS1 A190T is a resistant mutation of thiopurine by avoiding ADP or GDP inhibition

Activating PRPS1 A190T mutant can escape nucleotide feedback inhibition, so PRPS1 A190T mutant can be a resistant mutation of 6-mp in relapsed childhood ALL. To know whether the PRPS1 A190T mutant unlike PRPS1 WT showed the same drug resistance in BL as in ALL, we established the PRPS1 A190T mutation in BL and ALL cell lines (Figure 4A). We found that the PRPS1 A190T mutant presented...
Figure A shows Western blot analysis of PRPS1 and PRPS2 in control, PRPS1-WT-His, PRPS2-WT-Flag, PRPS1-WT-Flag, PRPS1-KO, and PRPS2-KO cells. The figure indicates that PRPS1 and PRPS2 expression levels are altered in these cell lines.

Figure B presents a bar graph showing the IC50 values (μg/mL) of 6-MP for different cell lines: Control PRPS1-WT-His, PRPS1-WT, PRPS2-WT, PRPS1-KO, and PRPS2-KO. The IC50 values are significantly different among the cell lines, with PRPS2-KO cells being the most sensitive to 6-MP.

Figure C displays a heatmap of metabolites measured in PRPS1-WT and PRPS2-WT cells treated with Vocb6. The heatmap shows the relative expression levels of various metabolites, with colors indicating the concentration levels.

Figure D displays Western blot analysis of PRPS1 and PRPS2 in control, PRPS1-WT-Flag, PRPS1-WT-Flag, PRPS1-KO, and PRPS2-KO cells. The figure indicates that PRPS1 and PRPS2 expression levels are altered in these cell lines.

Figure E presents a bar graph showing the IC50 values (μg/mL) of 6-MP for different cell lines: Control, PRPS1-WT, PRPS2-WT, PRPS1-KO, and PRPS2-KO. The IC50 values are significantly different among the cell lines, with PRPS2-KO cells being the most sensitive to 6-MP.

Figure F displays a heatmap of metabolites measured in PRPS1-WT and PRPS2-WT cells treated with Vocb6. The heatmap shows the relative expression levels of various metabolites, with colors indicating the concentration levels.

Figure G shows the IC50 values (μg/mL) of 6-MP for different cell lines: Control and HX(50 uM) treated cells. The IC50 values are significantly different among the cell lines, with HX(50 uM) treatment increasing the sensitivity to 6-MP.

Figure H displays Western blot analysis of PRPS1 and PRPS2 in control, PRPS1-WT-Flag, PRPS1-WT-Flag, PRPS1-KO, and PRPS2-KO cells. The figure indicates that PRPS1 and PRPS2 expression levels are altered in these cell lines.
6-mp drug resistance in cell lines with different metabolic levels (Figure 4B). In agreement with this, the purine metabolic level of A190T mutant cells was much higher (Figure 4C). Moreover, the relative thiopurine resistance in high metabolic cell lines was much lower than that in low metabolic cell lines (Figure 4B). Then, we tested the different concentrations on the 6-mp IC50 of PRPS1-A190T mutant cells, and we found that the 6-mp IC50 of PRPS1-A190T mutant cells decreased at increasing concentrations of ADP (Figure 4D). PRPS1-A190T in low metabolic cell lines could escape higher concentration of ADP than PRPS1-A190T in high metabolic cell lines (Figure 4D). These data suggest that the concentration of ADP/GDP is important to the thiopurine resistance. When the concentration is high enough, ADP/GDP can also inhibit the function of PRPS1-A190T. In a word, the thiopurine resistance of A190T mutations is limited.

3.5 | The combination of thiopurines and lometrexol is benefit to BL treatment

It has been reported that HD MTX and Ara-C are the major drugs in BL. Furthermore, our unpublished clinical trials put forward a new B-NHL treatment programme called the CCCG-B-NHL-2015 programme with HD MTX and ARA-C (Tables 1 and 2). In the mid-term summary, the B-NHL-2015 programme had a 2-year event-free survival rate (EFS) of 89.7 ± 1.8% compared to 76.1 ± 4.3% of the CCCG-BNHL-2010 programme (Figure 5A). Then, we used the LDH level of the B-NHL patients to indicate the dose of c-Myc expression. When the LDH level of the B-NHL patients was less than 4 times of the normal value (4N), increasing the dose of MTX and Ara-c could obviously prolong the 2-year EFS. However, when LDH was higher than 4N, the B-NHL-2015 programme showed no significant difference from the B-NHL-2010 programme (Figure 5B). All these clinical data suggest that the therapy research targeting for its metabolic pathway is clinically reasonable and applicable.

Lometrexol (also called DDATHF) which is one of the GART inhibitors has been tested as an antitumour drug. We firstly detected the IC50 of lometrexol in BL and ALL cells to choose the appropriate concentration (Figure 5B) and then did combination studies. According to our results, the lometrexol treatment dramatically decreased the thiopurine resistance in BL cells to ALL cells level (Figure 5C). Next, we tested the nucleotide metabolic level after adding the combination of 6-mp and lometrexol in BL and ALL cells. Compared to adding thiopurine only, the combination of thiopurine and lometrexol showed obviously lower

**TABLE 1** The CCCG-B-NHL-2015 and CCCGB-NHL 2010 programmes

| Regimen        | Risk group | Courses | CTX g/m² | IFOS g/m² | DOX mg/m² | MTX g/m² | ARA-C g/m² | VP-16 mg/m² | PRED/DEX mg/m² | VCR/VDS mg/m² | Lt. |
|----------------|------------|---------|----------|-----------|-----------|----------|-----------|-------------|----------------|----------------|-----|
| CCCGB-NHL 2010 | G3         | P+6     | 4.5      | 18        | 120       | 9        | 7         | 540         | 2835/—         | 18/—           | 13  |
|                | G4         | P+6     | 3.1      | 12        | 140       | 6        | 4         | 960         | 1995/—         | 12/—           | 13  |
| CCCG-B-NHL 2015| R3         | P+6     | 4.5      | 18        | 120       | 15       | 12        | 900         | 2835/—         | 3/15           | 13  |
|                | R4         | P+6     |          |           |           |          |           |             |                |                | 13/15 |

| Regimen | Stage and treatment protocol in the CCCG-B-NHL-2015 |
|---------|-----------------------------------------------------|
| R1      | Stage I, II, resected                                |
| R2      | Stage I, II, unresected, and LDH<2N                  |
| R3      | Stage III and LDH<4N; Stage I, II, LDH≥2N<4N         |
| R4      | Stage III and LDH≥4N; Stage IV; B-AL                |

**TABLE 2** The stage and treatment protocol in the CCCG-B-NHL-2015
metabolic levels in BL and ALL cells (Figure 5D). What's more, we observed that the lometrexol treatment could also decrease the thiopurine resistance in PRPS1 A190T mutant cells (Figure S1C). The IC50 of 6-mp in normal human cells like HF cells was much higher than the concentration we used in cancer cells (Figure S1D). Simultaneously, the IC50 of the combination treatment in HF cells was much higher than that in cancer cells (Figure S1D). Therefore, this combination did not have any added toxic effects on normal cells. These data suggested that the combination of 6-mp and lometrexol might serve as a therapeutic strategy to overcome thiopurine drug resistance in BL.

4 | DISCUSSION

Patients with relapsed/refractory BL have a dismal prognosis. Current research efforts aim to increase cure rates by identifying high-risk patients in need of more intensive or novel therapy. c-Myc gene which is typically overexpressed in BL can regulate the metabolic changes of tumour cells by driving PRPS2. Our present study is to gain a better understanding of the roles of c-Myc and PRPS1/2 in thiopurine drug resistance in BL to identify a more suitable molecular target and potentially improve the treatment of BL. Eventually, we found that the combination of thiopurines and the GART inhibitor lometrexol could overcome thiopurine drug resistance in BL which offered a new therapeutic strategy for the treatment of BL.

c-Myc which encodes an important transcription factor affects many physiological processes such as cell proliferation, growth and metabolism by activating multiple target genes. The Mannava's laboratory found that knocking out the c-Myc gene in human melanoma cell lines could reduce the expression of several rate-limiting enzymes during nucleotide synthesis, such as thymidylate synthase, HX nucleotide dehydrogenase 2 (IMPDH2) and PRPS2 resulting in a decrease in intracellular nucleotide levels and cell proliferation. Moreover, in c-Myc overexpressed cancer cells, the activated eukaryotic initiation factor 4E could bind with the pyrimidine-rich translational element of PRPS2 mRNA to promote the translation of PRPS2 making the level of nucleotide metabolism up-regulated. However, when we directly regulated the level of c-Myc, the level of nucleotide metabolism was unchanged while the regulation had no effects on the resistance to thiopurine drugs. Considering the results we have achieved, it seems that c-Myc cannot significantly regulate the nucleotide metabolism so it does not have direct relationship with the resistance to thiopurine drugs.

Phosphoribosyl pyrophosphate synthetase 1 and PRPS2 are homologous isoenzymes that catalyse the same biochemical reaction in both nucleotide de novo synthesis and salvage synthesis pathway. In 1995, Tatibana et al found that the recombinant expression of PRPS1 protein in vitro existed in the form of stable hexamers which could be significantly inhibited by ADP and GDP. Moreover, PRPS2 has lower enzymatic and allosteric activity than PRPS1, but is sensitive to cytokine regulatory responses. Consistently, our study showed that in BL cells, the function of PRPS1 was greatly inhibited by the high produced nucleotide metabolite like ADP and GDP. Overexpression of PRPS1 showed no influences on resistance to 6-mp. On the contrary, in ALL cells with low metabolic level, PRPS1 with low ADP, GDP inhibition performed the key role in the thiopurine resistance. We also noticed that in either high or low metabolic cells, PRPS2 had almost no function on the thiopurine resistance for its low enzymatic and allosteric activity. And more, the regulation of PRPS1/2 in Nalm6 cell line did not obviously affect the sensitivity to thiopurine drugs. These data suggest that mutations affecting DNA stability can mask the effects of PRPS on drug resistance.

Phosphoribosyl pyrophosphate synthetase synthetase 1 A190T mutant which is a negative feedback-defective PRPS1 mutant can be a resistant mutation of 6-mp in relapsed childhood ALL. Although the A190T mutant showed significantly 6-mp resistance in BL, the IC50 to thiopurine resistance was much lower than that in ALL cells. These data suggest that the PRPS1 A190T mutant can be inhibited if the level of nucleotide metabolism is high enough.

The French-American-British (FAB) Lymphomes Malins B studies demonstrated that HD MTX was an effective drug for CNS prophylaxis in BL. The obligatory Berlin-Frankfurt-Munster (BFM) studies also showed that the LDH level as c-Myc marker was an important factor in the treatment of BL. The ongoing CCG-B-NHL-2015 confirmed those conclusions by increasing the dose of anti-metabolic chemotherapeutic drugs. However, there remains a subgroup of patients with LDH >4N for whom novel treatment approaches are needed. In our study, the GART inhibitor lometrexol can increase the sensitivity of BL cells to thiopurines by inhibiting the nucleotide metabolism.

In summary, here we have reported a distinct role of c-Myc and PRPS1/2 in thiopurine drug resistance in BL. Combination of thiopurines and the GART inhibitor lometrexol can overcome thiopurine drug resistance in BL. Therefore, our results not only clarify the role of c-Myc and PRPS1/2 in the treatment of BL, but also offer a new therapeutic strategy for the treatment of BL.
A

Event-free survival (proportion)

0  0.2  0.4  0.6  0.8  1.0

Time (mo)

CCCG-BNHL-2015 2-year EFS 92.1 ± 1.6%
CCCG-BNHL-2010 2-year EFS 81.6 ± 4.1%

B

EFS at 2 years (%)

CCCG-BNHL-2010
CCCG-BNHL-2015

LDH<2N  2N=LDH<4N  LDH>4N

C

IC50 (μg/mL)

Lometrexol (0 ng/mL)
Lometrexol (5 ng/mL)

Raji  Molt4

D

Raji  Molt4

6-MP  6-MP+ Lometrexol

Urate  IMP  HX  AMP  ADP  GDP  UDP  ATP  GTP  dAMP  dGMP  dADP  dCDP  dTDP  dGTP  dATP  dTTP
CONFLICT OF INTEREST
The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS
This study was conceived by YJ G and YX L.; YJ G and YX L. designed the study; T. L., LL S., YL, ZY Z, YW Z, YY, SQ W and SS L performed the experiments; T. L. analysed and interpreted the data; YJ G reviewed the manuscript; and TL and YX L. wrote the paper with comments from all authors. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT
All data supporting the findings of this study are available from the corresponding author on request.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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