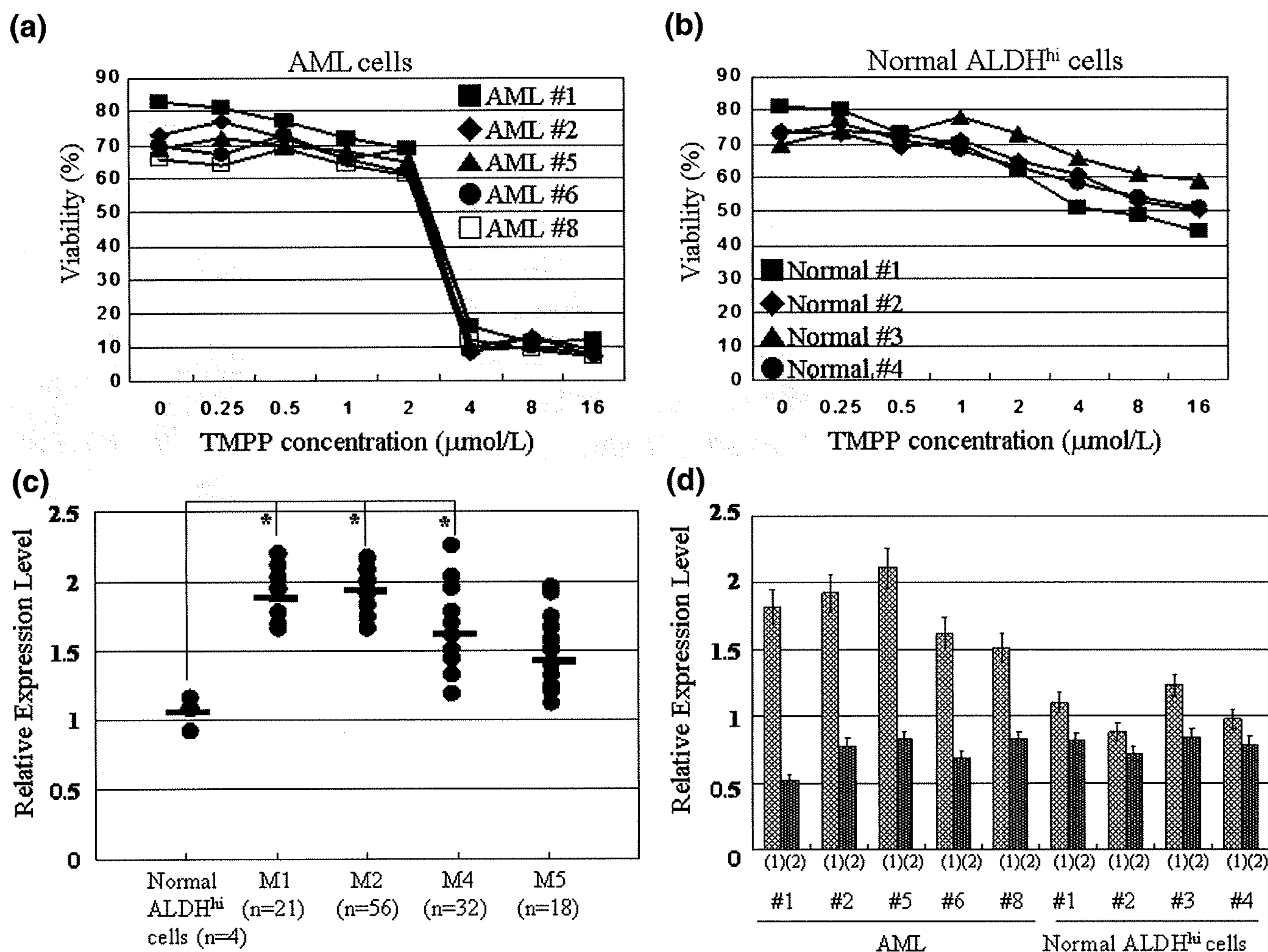

Fig. 6 Effect of TMPP treatment on AML cells and ALDH^{hi} hematopoietic progenitor cells, and effect of overexpression of FoxM1 on AML cell proliferation. AML cells derived from AML patients (M2; #1, #2, #5, M4; #6, #8) (**a**) and ALDH^{hi} cells derived from healthy volunteers (n=4) (**b**) were treated with TMPP at the indicated concentrations for 48 h. (**c**) FoxM1 mRNA expression levels in primary AML cells. (**d**) AML cells derived from AML patients (M2; #1, #2, #5, M4; #6, #8) were treated with 5 μM TMPP for 48 h. After treatment, the cells were harvested and Quantitative RT-PCR was performed for measurement of FoxM1 mRNA. (1) untreated, (2) treated with TMPP. Data are shown as mean±S.D. in triplicate culture and are representative of three independent experiments. *P<0.05 comparing FoxM1 mRNA expression

(Fig. 6(d)). FoxM1 expression in AML cells showed mean 28.6% (#1), 40.2% (#2), 38.9% (#5), 42.0% (#6), and 54.3% (#8) inhibition, relative to untreated cells. In contrast, the normal ALDH^{hi} cells showed mean 75.8± 6.75% expression inhibition at 5 μM TMPP for 48 h. These results indicate that FoxM1 mRNA were overexpressed in acute leukemia specimens, and reduction of FoxM1 mRNA by TMPP treatment inhibited cell proliferation.

Discussion

Here, we synthesized two phospha sugar derivatives, TMPP and DMPP, and evaluated their antileukemic effects in AML cell lines, AML cells derived from AML patients, and normal hematopoietic progenitor cells *in vitro*. Results showed that both TMPP and DMPP suppressed the proliferation of leukemia cells by downregulation of FoxM1, FoxM1, which is a member of the forkhead box family, is required for coupling DNA replication with mitosis, expression, at least in part, leading to G2/M cell cycle block and subsequent caspase-3-dependent apoptosis. Further, we identified FoxM1 as a target molecule of therapeutic regimens for leukemia patients. Relative to other therapeutic agents, TMPP/DMPP represent a structurally distinct type of small-molecule agent with unique mechanistic features.

TMPP and DMPP were found to have antileukemic effects in AML cell lines (mean IC_{50} 6.25 μmol/L and 23.7 μmol/L, respectively). In the MTT assay, TMPP more strongly inhibited the proliferation of leukemia cells than DMPP, an effect attributable to TMPP having more bromine atoms than DMPP. Further, concentrations of 10 μmol/L TMPP and

20 μmol/L DMPP both induced G2/M cell cycle block, while 20 μmol/L TMPP additionally induced apoptosis in leukemia cells. Moreover, TMPP treatment induced a decrease in cell cycle progression signals, tumor cell survival, and led to the activation of caspase-3 and -9.

FoxM1 is a transcription factor that regulates proliferation and cell cycle progression. Recently, it has been reported that FoxM1 promotes cell cycle progression by downregulation $p27^{Kip1}$ via multiple mechanisms [23]. We also have reported previously that overexpression of FoxM1 regulates the proliferation of leukemia cells, suggesting a potential mechanism for cell cycle progression in leukemia cells (in press). In current leukemia therapy, however, there are no effective antileukemic agents which target transcription factors.

Surprisingly, we found that TMPP/DMPP suppressed FoxM1 expression in leukemia cells. Although information regarding the effect of FoxM1 down-regulation in leukemia cells is scarce, we found that reducing FoxM1 expression resulted in both the inhibition of leukemia cell proliferation and an increase in the population of leukemia cells at G2/M phase 7 days after transfection compared to control siRNA-transfected cells and untreated cells. In contrast, overexpression of FoxM1 promoted the proliferation of leukemia cells. These data are consistent with the notion that FoxM1 protein is a key regulator of G2/M progression and is activated in many human malignancies [23–29]. Further, we showed that FoxM1 reduction not only increased the expression of $p27^{Kip1}$ and $p21^{Cip1}$ proteins, but also decreased that of Cdc25B, Cyclin D1, Cyclin A, KIS, and Aurora-B kinase proteins. Further, the decrease in expression of Cdc25B, Cyclin D1, Cyclin B, KIS, and Aurora-B kinase and the increase in expression of $p27^{Kip1}$ and $p21^{Cip1}$ proteins were strongly correlated with the altered cell cycle distribution and leukemia cell growth suppression. These results suggest that FoxM1 affects the leukemia cell cycle by regulating the expression levels of these proteins and may represent for a potential target for TMPP treatment.

FoxM1 has been reported to affect both the G1-S and G2-M transitions in the cell cycle [30]. G1-S transition is promoted by suppression of CDKIs such as $p27^{Kip1}$ brought on by inducing expression of KIS and Cyclin D1. G2-M transition is promoted via suppression of CDKIs such as $p21^{Cip1}$ brought on by inducing expression of Cdc25B, Cyclin A, and Aurora-B kinase. In the present study, treatment with low concentrations of TMPP and DMPP resulted in an increase in the distribution of G2/M, but not G1, leukemia cells, and no increase in the population of apoptotic cells. On western blot analysis, we found a greater reduction in levels of Cdc25B, Cyclin A, and $p21^{Cip1}$, which relate to the G2-M transition process, than in those of KIS, Cyclin D1, and $p27^{Kip1}$, which relate to the G1-S transition process. These results may suggest the mechanisms of the increase of G2/M distribution.

Further, TMPP was shown to act on expression of several key cell cycle proteins to cause arrest or apoptosis in a dose-dependent manner. Several studies have found that expression of $p21^{Cip1}$ causes strong G2 arrest and induces resumption of leukemia cell cycle progression [31, 32]. In leukemia cells, there is a strong correlation between the expression of Cyclin D1 and $p21^{Cip1}$, which reflects a complicated network regulating the proliferation and differentiation of these cells [33]. Aurora-B kinase has been reported to be aberrantly expressed in several human leukemia cell lines (n=15, e.g. PALL-1, PALL-2, HL-60, NB4, MV4-11, etc) as well as in freshly isolated leukemia cells from individuals with AML (n=44) [34]. Here, we found that TMPP treatment at low concentration reduced Aurora-B kinase expression in leukemia cells. At high concentration, TMPP induced apoptosis of leukemia cells via the caspase pathway. These results suggest that TMPP induced an increase in the G2/M cell population at a low concentration and an increase in apoptotic cells at a high concentration in leukemia cells.

The cytotoxic effects of TMPP on the cell lines above were also seen in clinical AML specimens derived from AML patients. The viability of AML cells was significantly reduced at TMPP concentrations greater than 4 μmol/L, whereas the viability of normal $ALDH^{hi}$ cells was only slightly reduced with TMPP administration in a dose-dependent manner. Levels of CFU-GEMM (colony forming unit-granulocyte, erythroid, macrophage, megakaryocyte), CFU-GM (colony forming unit-granulocyte, macrophage) and BFU-E (burst forming unit-erythroid) derived from normal progenitor cells were moderately reduced on treatment with TMPP compared to untreated cells (data not shown). Moreover, we observed that FoxM1 mRNA was overexpressed in acute leukemia specimens, and treatment with TMPP reduced the expression of FoxM1 mRNA an AML cells. Taken together, these data indicate that inhibition of FoxM1 represents an attractive target for leukemia therapy.

In conclusion, we synthesized two deoxybromophospha sugar derivatives, TMPP and DMPP, and found that these agents are potent inhibitors of FoxM1, inducing G2/M cell cycle block via down-regulation of FoxM1 at low concentrations, and apoptosis via the caspase pathway at high concentrations *in vitro*. FoxM1 has therefore been characterized as a target molecule of TMPP in leukemia cells. Further, TMPP significantly reduced AML cell viability in clinical specimens derived from AML patients, but not in normal hematopoietic progenitor cells. These results suggest that TMPP may efficiently inhibit cell cycle and induce apoptosis, and thereby facilitate the development of new strategies in targeted antileukemic therapy.

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