

Glucosylceramide Synthase Is a Novel Biomarker of Midostaurin-Induced Cytotoxicity in Non-Mutant FLT3 Positive Acute Myeloid Leukemia Cells

Glukozilseramid Sentaz Mutant Olmayan FLT3 Pozitif Akut Miyeloid Lösemi Hücrelerinde Midostaurin İlişkili Sitotoksitenin Yeni Bir Biyobelirtecidir

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ABSTRACT

Objective: Glucosylceramide (GC) synthesized by glucosylceramide synthase (GCS) favors cell survival and proliferation in many cancers. However, its role in Fms-like tyrosine kinase 3 (FLT3) non-mutant Acute Myeloid Leukemia (AML) pathogenesis is not clarified. Midostaurin, a multi-kinase inhibitor, clinically benefits FLT3-mutated AML, however, its clinical efficacy is under-estimated in FLT3 non-mutant AML. This study aimed to investigate the efficacy of combination of midostaurin with GCS inhibitor in FLT3 AML cell carrying wild-type FLT3 and the underlying molecular mechanisms.

Material and Method: Cytotoxic and cytostatic effects of midostaurin, PDMP (GCS inhibitor) alone and in combination on THP1 cells were determined by MTT assay and flow cytometric propidium iodide (PI) staining, respectively. Calcsyn software was used to calculate combination indexes (CIs). GCS expression was checked by western blot.

Results: Midostaurin downregulated GCS. Simultaneous inhibition of FLT3 and GCS resulted in suppression of cell proliferation as compared to untreated control. Combinations showed synergistic cytotoxic effects (CI<1). Co-treatments increased cell cycle population at G2/M phase.

Conclusion: Inhibition of GCS enhances the efficacy of midostaurin in FLT3 non-mutant AML, which could be a novel therapeutic approach to increase midostaurin's limited usage in the clinic after detailed mechanistic studies.

Keywords: Cell cycle, FLT3 non-mutant AML, glucosylceramide synthase, midostaurin

ÖZ

Amaç: Glukozilseramid sentaz (GSS) tarafından sentezlenen glukozilseramid (GS) birçok kanser türünde hücre yaşamını ve proliferasyonunu sağlamaktadır. Ancak, mutant olmayan Fms-benzeri tirozin kinaz 3 (FLT3) pozitif akut miyeloid lösemi (AML) patogenezindeki rolü açıklanmamıştır. Çoklu kinaz inhibitörü olan midostaurin mutant FLT3 AML tedavisinde etkili olmasına rağmen mutant olmayan FLT3 pozitif AML'deki klinik etkisi gözden kaçırılmıştır. Bu çalışmada, midostaurinin GSS inhibitörü ile kombinasyonunun yabancı tip FLT3 ifadesine sahip AML hücrelerindeki etkisinin belirlenmesi ve moleküler mekanizmalarının açıklanması amaçlanmıştır.

Gereç ve Yöntem: Midostaurin, PDMP (GSS inhibitörü) ve kombinasyonların THP1 hücreleri üzerindeki sitotoksik ve sitostatik etkileri sırasıyla MTT testi ve PI boyaması ile akım sitometri kullanılarak belirlenmiştir. Kombinasyon indeksleri (CI) Calcsyn programı ile hesaplanmıştır. GSS ifadesi western blot ile belirlenmiştir.

Bulgular: Midostaurin GSS ifadesini baskılamıştır. FLT3 ve GSS'in birlikte inhibe edilmesi kontrolle karşılaştırıldığında hücre çoğalmasını baskılamıştır. Kombinasyonlar sinerjistik sitotoksik etki göstermiştir (CI<1). Kombinasyon hücre döngüsünün G2/M fazındaki hücre popülasyonunu arttırmıştır.

Sonuç: Mutant olmayan FLT3 AML'de GSS inhibisyonunun midostaurin'in etkisini arttırdığı saptanmıştır. Detaylı mekanizma çalışmaları yapıldıktan sonra kombinasyon tedavisinin midostaurin'in sınırlı klinik kullanımını artırması açısından yeni bir yaklaşım olabileceği düşünülmektedir.

Anahtar Kelimeler: Hücre döngüsü, FLT3 mutant olmayan AML, glukozilseramid sentaz, midostaurin

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INTRODUCTION

Acute myeloid leukemia (AML) is a genetically complex disorder, which results in different prognostic subgroups (1). Fms-like tyrosine kinase 3 (FLT3) gene is considered as the most important one among the altered genes in AML, leading to clinically problematic FLT3 positive AML subgroups in both newly diagnosed adult patients and pediatric AML (2).

The FLT3 gene is included in the receptor tyrosine kinase family and commonly found on the plasma membrane of hematopoietic stem and early progenitor cells and over-expressed or mutated on a high percentage of AML blasts (3). Dimerization and activation of FLT3 is induced after binding of FLT3 ligand, which leads to activated downstream RAS, PI3K/AKT and STAT5 signaling pathways involved in cell proliferation and inhibition of apoptosis (3,4). FLT3 receptor and its downstream pathways become constitutively active as a result of two common FLT3 mutations, which are found in tyrosine kinase domain (TKD) and within its juxtamembrane domain (internal tandem duplication, ITD) (2,4). Therefore, development of new small molecule inhibitors has dramatically changed the treatment course of FLT3 positive AML after defining the critical role of FLT3 signaling in disease pathogenesis. FLT3 inhibitors include first generation inhibitors such as midostaurin and sorafenib and next generation inhibitors such as crenolanib and gilteritinib, which differ in their specificity and potency (5). Among these inhibitors, midostaurin (PKC412) was the first one clinically approved for the treatment of newly diagnosed FLT3-mutation carrying patients in combinations with standard treatment protocols (6). However, some clinical trials revealed that midostaurin is also effective in non-mutant FLT3 positive AML, since 70-100% of AML patients overexpress FLT3 and it has multiple cellular targets in addition to FLT3 (7,8). Molecular studies investigating this transient effect of midostaurin in non-mutant FLT3 AML are very scarce, which clearly indicated that FLT3-independent molecules or pathways might have a role to regulate its efficacy. In a study, ERK and KIT kinases have been shown to be targeted by midostaurin and their pharmacological inhibition enhanced its effect (9). In another study, MEK was identified as a midostaurin target and co-inhibition of MEK and FLT3 gave promising results (10). Therefore, we suggest that one of the mechanisms contributing to midostaurin's efficacy in non-mutant FLT3 AML could be the glucosylceramide synthase (GCS) enzyme involved in ceramide (Cer) catabolism, which has many therapeutic targets in different cancers.

Sphingolipids including Cer and its phosphorylated or glycosylated forms determine the cell fate, which is either cell survival or cell death (11). Members of the sphingolipid family affect cancer initiation and progression by modulating cell growth, and division, apoptosis, metastasis and response to therapy (12). The central molecule Cer derived is known to be an apoptotic lipid whilst its glycosylated form glucosylceramide (GC) is involved in cell proliferation and drug resistance (11,13). Cer produced via *de novo* or salvage pathway is transferred to *cis*-Golgi to be glycosylated by GSC, hence this enzyme with its anti-carcinogenic activities is shown to possess therapeutic importance in cancers

(14). Inhibition of GCS in head and neck cancer overcame resistance to cisplatin by downregulating p-glycoprotein and upregulating apoptotic proteins (15). In Chronic Myeloid Leukemia (CML) cells, GCS was found overexpressed in imatinib-resistant cells as compared to sensitive partners and targeting GCS sensitized resistant cells to imatinib (16). Delivery of Cer nanoliposomes plus simultaneous inhibition of GCS resulted in induction of the intrinsic pathway of apoptosis in natural killer cell leukemia (17). Therefore, investigation of the involvement of GCS in midostaurin's effect on non-mutant FLT3 AML could open a new route to understand midostaurin's observed clinical activity and provide a novel target.

In this study, we hypothesized that inhibiting GCS in combination with midostaurin would enhance midostaurin's antileukemic activity in THP1 cells for the first time, and we could suggest a possible combination approach.

MATERIAL AND METHOD

Chemicals and Agents

MTT and midostaurin were commercially supplied (Sigma-Aldrich, USA). 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) was obtained from Cayman Chemicals (Ann Arbor, MI, USA). Stock solutions (10 mM in dimethyl sulfoxide (DMSO)) were prepared, which do not contain more than 0.01% DMSO in culture. RPMI 1640 growth medium and all ingredients required for complete growth medium (Penicillin-streptomycin and fetal bovine serum) were obtained from Invitrogen (Paisley, UK).

Cell Lines and Culture Conditions

THP1 cell line, representing non-mutant/wild type FLT3 carrying AML cell line, was purchased from German Collection of Microorganisms and Cell Cultures (Germany, DSMZ) and cultured and maintained in RPMI-1640 growth medium (with L-glutamine) including 10-20% fetal bovine serum (FBS) and 1% penicillin-streptomycin up to 15 passages at 37°C in a 5% CO₂ incubator.

Cell Viability Assay

Cell viability was analyzed by the standard MTT protocol in response to increasing concentrations of midostaurin (100-1000 nM), and PDMP (1-80 µM) on THP1 cells (18). 1x10⁴ cells/well were seeded in 96-well plates for 48 h. Following treatments, a 20 µl MTT solution (5 mg/mL, Sigma Aldrich) was added. Then, absorbances were recorded at 570 nm using a spectrophotometer. Based on the cell proliferation graphs, IC₅₀ values (concentration inhibiting cell growth by 50%) for midostaurin and PDMP were calculated by linear regression analysis using GraphPad software (San Diego, CA).

Combination Index (CI) Analysis

Midostaurin (200-800 nM) was combined with PDMP (10-40 µM) at a fixed molar ratio. After 48 h of exposure, cell survival was assessed by the standard MTT assay. The method of Chou and Talalay was used to define whether combinations lead to synergism (CI<1), additivity (CI:1.0-1.1) or antagonism (CI>1.1) using CalcuSyn software (Biosoft, Cambridge, United Kingdom) (19,20).

Cell Cycle Analysis

7.5×10^5 cells/2ml were treated with midostaurin (400-800 nM), PDMP (20-40 μ M) and their combinations (synergistic combinations were chosen based on CI analysis) for 48 h. Then, the cells were fixed with cold ethanol overnight at -20°C . The cells were treated with RNase-A (200 μ g/ml, Sigma Aldrich) at 37°C for 30 min and then with propidium iodide (PI) (1 mg/ml, Sigma Aldrich) at room temperature for 10-15 minutes (18). Cell cycle analysis was carried out by BD FACSCalibur flow cytometer (BD Biosciences). The results were analyzed using BD FACSDiva™ (BD Biosciences).

Western Blot Analysis

5×10^6 cells were incubated with midostaurin (200 and 400 nM) for 48 h to detect the changes in the expression of GCS by western blot. After cell lysis in the RIPA buffer (Sigma-Aldrich, USA), protein amounts were detected with RC DC™ Protein Assay Kit (Bio-Rad, USA). 30 μ g protein was loaded to SDS-PAGE and separated proteins were transferred to PVDF membranes. Primary antibodies for GCS (1:3000, Novus Biologicals, USA) and Beta Actin (1:3000, Cell Signaling, USA) were used to detect the proteins and conjugated with secondary antibodies (1:10000, Jackson ImmunoResearch, USA). The protein bands were visualized with Pierce™ ECL Western Blotting Substrate kit (Thermo Scientific™, USA). Densitometric analysis of immunoreactive bands was carried out by the imaging software (Bio-Rad, ChemiDoc, Image Lab™ 3.0).

Statistical Analysis

Three independent experiments were done where the results are expressed as mean \pm standard deviation (SD). GraphPad Prism 6.0 was used for statistical analysis. One-way ANOVA was applied followed by Dunnett's or Tukey's multiple comparisons tests. $p < 0.05$ was accepted as statistically significant.

RESULTS

Midostaurin Decreased Glucosylceramide Synthase Level in THP1 Cells

Changes in GCS protein level in response to sub-toxic concentrations of midostaurin (200 and 400 nM) were analyzed.

400 nM midostaurin caused a 0.5-fold decrease in GCS expression compared with the untreated control group (Figure 1) although 200 nM midostaurin did not affect GCS expression significantly. Therefore, it could be suggested that GCS might be a critical enzymatic target involved in non-mutant FLT3 positive AML pathogenesis.

Co-inhibition of FLT3 and GCS Exerted Synergistic Cytotoxic Effect in THP1 Cells

It was previously shown that midostaurin alone inhibited the viability of THP1 cells with an IC_{50} : 916 nM (Figure 2A) (21). PDMP alone did not affect leukemic cell viability significantly up to 40 μ M and its IC_{50} was approximately 63 μ M (Figure 2B). To determine whether the inhibition of GCS by PDMP could affect anti-proliferative action of midostaurin, THP1 cells were treated with midostaurin plus PDMP by using their sub-toxic concentrations for 48 h. As shown in Figure 2C, at midostaurin concentrations of 400 nM and 800 nM, viability of THP1 cells exposed to 20 and 40 μ M PDMP was significantly inhibited as compared to the control. (Figure 2C). Viability of the cells treated with 800 nM midostaurin in combination with 40 μ M PDMP was significantly lower than that of the cells treated with 800 nM midostaurin alone (Figure 2C). CIs were also calculated (Figure 2D). The combination of 400 and 800 nM midostaurin with 20 and 40 μ M PDMP, respectively resulted in synergistic toxicity (CIs: 0.6 and 0.8)

Co-targeting FLT3 and GCS Arrested THP1 Cells at G2/M Phase

To determine whether the enhanced midostaurin toxicity in the presence of PDMP was related to changes in distribution of cell cycle populations, we analyzed the cell cycle percentages of the cells treated with midostaurin and PDMP alone or their combinations based on CalcuSyn analysis. 400 and 800 nM midostaurin alone blocked cell cycle progression at the G2/M phase (20.1 and 36.4 vs 9.5%, respectively) as shown in the previous study (21), whereas PDMP alone did not induce changes in any of the cell cycle phases as compared to control (Figure 3A and Figure 3B). Both combinations stopped the cell cycle at the G2/M phase (37 and 37.1 vs 9.5%, respectively) as

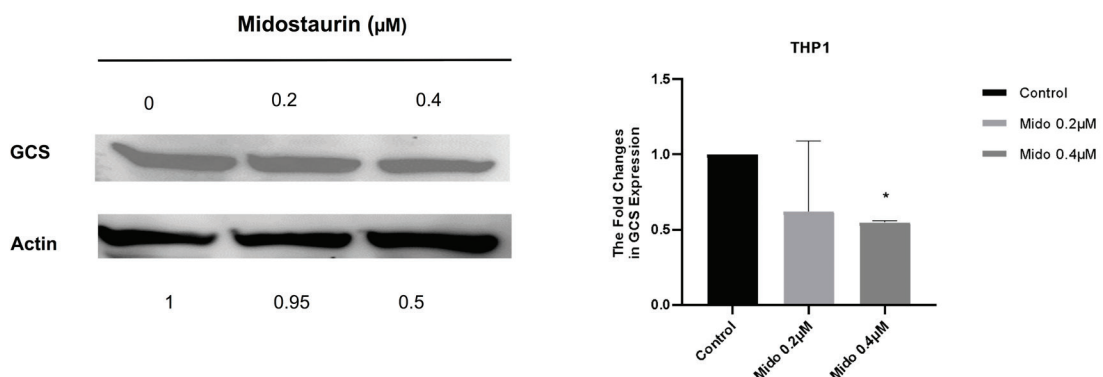


Figure 1. Western blot analysis of GCS expression in response to midostaurin in THP1 cells. The results from three independent experiments were given as mean \pm SD. * $p < 0.05$ vs. control. GCS: Glucosylceramide synthase; Mido: Midostaurin

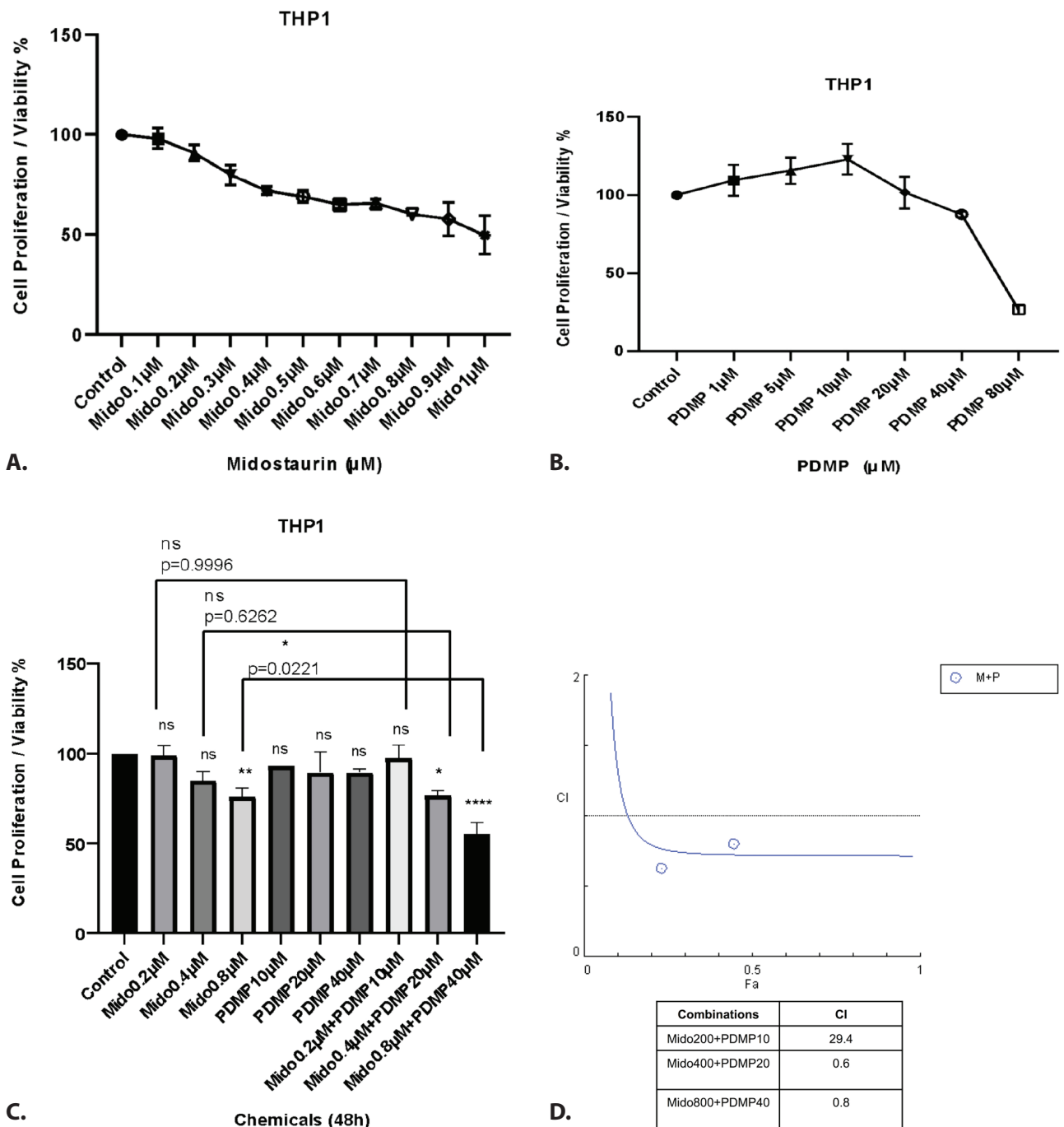


Figure 2. Anti-proliferative effects of midostaurin (A), PDMP (B) and combinations (C) in THP1 cells. Results are shown as means ± SD of three experimental setups performed at different times. Calculation of CI values based on the method of Chou and Talalay (D). *p<0.05, **p<0.005, ****p<0.0001 versus control. Mido: Midostaurin

compared to control. 400 nM midostaurin with 20 µM PDMP arrested the cells at G2/M phase as compared to midostaurin alone (20.1% and 37%, respectively) (Figure 3A and Figure 3B). These data further supported the enhancing effect of GCS inhibition on the cytotoxicity of midostaurin alone.

DISCUSSION

The roles of sphingolipid metabolism's intermediates including Cer and its modified forms in carcinogenesis and response to therapy have been extensively investigated (22). In this concept, we explored the role of GCS for the first time in mi-

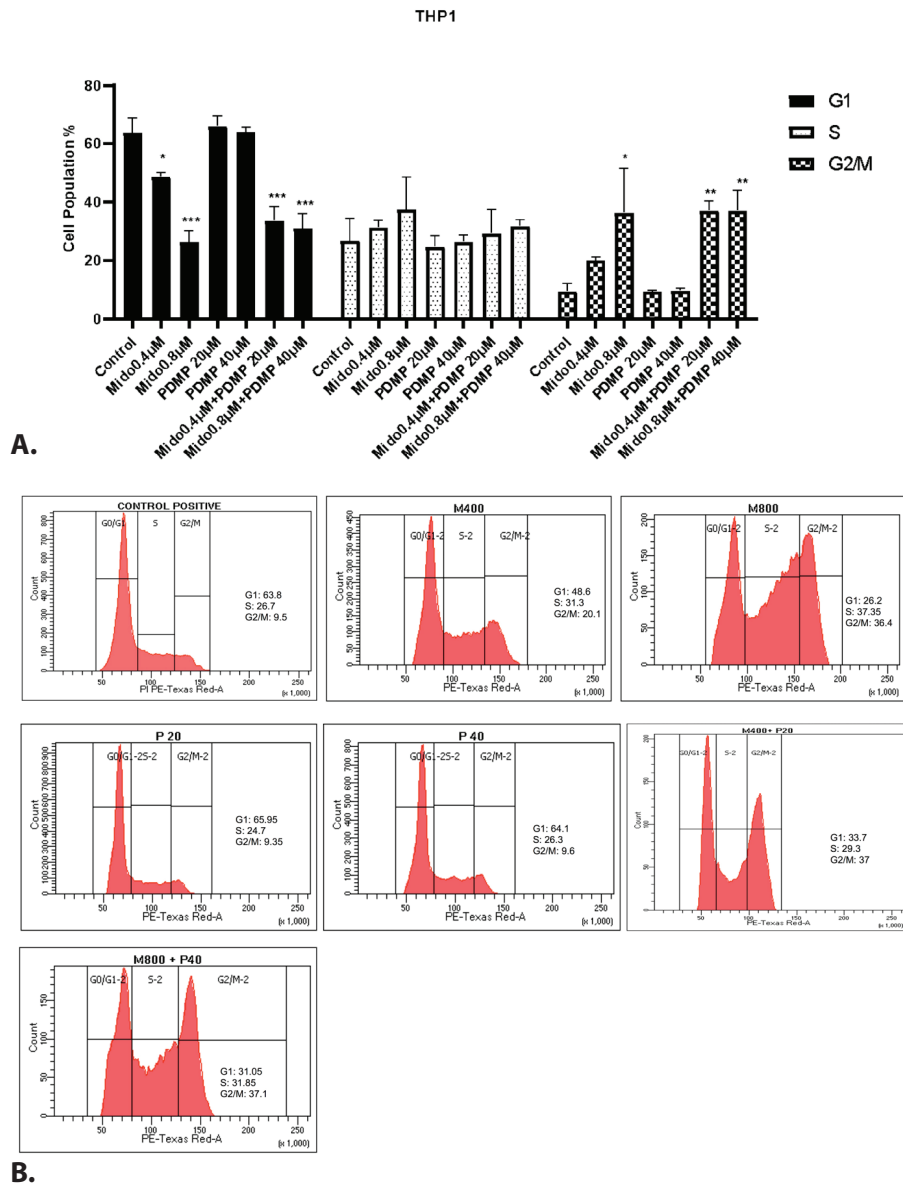


Figure 3. Cell cycle distribution of THP1 cells treated with midostaurin, PDMP and combinations. Data are shown as the means \pm SD of three experimental setups performed at different times. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$ versus control. Mido: Midostaurin

Midostaurin's anti-leukemic action in non-mutant FLT3 positive AML although its clinical approval has been granted for only patients carrying FLT3 mutations (23). Midostaurin's activity in non-mutant FLT3 AML patients was also encouraging based on a phase III trial (24), which could suggest the presence of FLT3-independent targets responsible for its broad activity as explained previously (9,10).

GCS resides in golgi apparatus and catalyzes the conversion of Cer into GC by transferring UDP-glucose to Cer. GC is shown to be overexpressed in various cancers and found to be associated with increased cell proliferation and failed treatment

response (14). In this study, we suggest that GCS could be associated with observed clinical response in phase studies and a new therapeutic target could be defined if its role in midostaurin's action is enlightened in non-mutant FLT3 positive AML.

The protein level of GCS was decreased after midostaurin treatment as compared to untreated cells (Figure 1) and untreated cells had also high GCS expression, suggesting that GCS could be involved in response to midostaurin treatment as well as having a role in non-mutant FLT3 positive AML pathogenesis. There are studies regarding the role of GCS in several hematological malignancies. In resistant chronic myeloid leukemias,

GCS was shown to be upregulated (16,25). Based on this result, THP1 cells were treated with midostaurin plus PDMP to determine the presence of any synergistic cytotoxic effect. The results suggested that midostaurin's growth inhibitory effect on THP1 cells was enhanced via showing synergistic inhibition of cell viability ($CI_s < 1$ for combinations) (Figure 2C and Figure 2D). Hence, GCS could be defined as a therapeutic target in THP cells, which is in accordance with the findings in several cancers. Inhibition of GCS by using a specific inhibitor together with chemotherapeutics sensitized resistant leukemia cells by increasing apoptosis (25). In prostate cancer cells, co-treatment with docetaxel and GCS inhibitor enhanced docetaxel's cytotoxic activity by inducing strong synergism (26). PDMP also potentiated the cytotoxic effect of dasatinib in CML cells with CI values lower than 1 (27).

To further investigate the enhanced cytotoxicity of midostaurin induced by PDMP, cell cycle populations were quantified after treatment with midostaurin and PMDP alone and in combinations. The results showed that PDMP alone did not cause changes at cell cycle phases, however midostaurin alone arrested the cells at G2/M (Figure 3). This cytostatic effect of midostaurin was in accordance with studies comparing the effects of midostaurin on cell cycle distribution between non-mutant and mutant FLT3 positive AML and FLT3 wild type AML (28,21). The studies related to cell cycle profiles of the leukemic cells treated with GCS inhibitors showed varying results based on the leukemia type. In K562 CML cells, PDMP exposure resulted in G1 arrest and it caused more accumulation together with imatinib (16). Another GCS inhibitor PPPP did not change cell cycle profiles of leukemic cells alone, however, combination with vincristine increased G2/M population (29). In this study, co-treatments induced G2/M arrest as compared to the control or midostaurin alone (Figure 3). There are very limited studies investigating the contribution of Cer or its metabolites to midostaurin's effect in cancer in which liposomal Cer treatment or sphingosine kinase-1 inhibition increased midostaurin's activity (30,21).

In conclusion, midostaurin's cytotoxic activity is enhanced in the presence of PDMP, which supported the involvement of GCS in midostaurin treatment and non-mutant FLT3 positive AML pathogenesis through inducing synergistic cell cycle arrest. Even though this preliminary study could suggest a new target of midostaurin, it is still needed to elucidate the relationship between midostaurin and GCS mechanistically. It would be suggested to investigate the role of signaling pathways leading to cell death and to analyze cell cycle regulators involved in G2/M phase regulation in response to co-treatments. Pre-clinical *in vitro* models, *in vivo* mouse studies and *ex vivo* patient samples could be studied to reveal the expression level of GCS after midostaurin treatment.

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