Research Article

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Combined effect of midostaurin and sphingosine kinase-1 inhibitor on FMS-like tyrosine kinase 3 (FLT3) wild type acute myeloid leukemia cells

FMS benzeri tirozin kinaz 3 (FLT3) yabanıl tip akut miyeloid lösemi hücrelerinde midostaurin ve sfingozin kinaz-1 inhibitörünün kombine etkisi

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Abstract

Objectives: Therapeutic potential of clinically approved FLT3 inhibitor midostaurin has been neglected in wild-type FLT3 positive acute myeloid leukemia (AML). Sphingosine kinase-1 (SK-1) having anti-proliferative functions is studied in various cancers, but not in FLT3 wild-type AML. We aimed to develop new therapeutic strategies to combat FLT3 wild-type AML by combining midostaurin with SK-1 inhibitor (SKI II) in THP1 cells.

Methods: The anti-proliferative effects of midostaurin, SKI II and in combination on THP1 cells were determined by MTT assay. The combination indexes were calculated using calcusyn software. SK-1 expression and PARP cleavage were checked by western blot. Cell cycle distributions (PI staining) and apoptosis (annexin-V/PI dual staining) were assessed by flow cytometry for each agent alone and in combinations.

Results: Midostaurin decreased SK-1 protein level. Midostaurin, SKI II and certain combinations decreased cell viability in a dose dependent manner. The combined anti-leukemic effects of the aforementioned drug combination afforded additive effect. Co-administration induced both necrosis and apoptosis via phosphatidylserine externalization, PARP cleavage and cell cycle arrest at G0/G1 and S phases.

Conclusions: Targeting sphingosine kinase-1 together with FLT3 inhibition could be a novel mechanism to increase limited clinic response to midostaurin in wild-type FLT3 overexpressing AML after further pre-clinical studies.

Keywords: apoptosis; FLT3 wild type AML; midostaurin; sphingosine kinase; sphingosine kinase inhibitor.

Öz

Amaç: Klinik olarak onaylanmış FLT3 inhibitörü olan midostaurinin yabanıl tip FLT3 pozitif akut miyeloid lösemide (AML)'deki terapötik potansiyeli ihmal edilmiştir. Anti-proliferatif rolleri olan sfingozin kinaz-1 (SK-1) bir çok kanserde çalışılmış fakat yabanıl tip FLT3 AML'deki rolü bilinmemektedir. Bu çalışmada, midostaurinin sfingozin kinaz-1 inhibitörü (SKI II) ile kombinasyonunun THP1 hücrelerindeki etkisinin belirlenmesi ile yeni bir tedavi yaklaşımının araştırılması amaçlanmıştır.

Yöntem: Midostaurin, SKI II ve kombinasyonların THP1 hücreleri üzerindeki anti-proliferatif etkisi MTT testi ile saptanmıştır. Kombinasyon indeksleri Calcusyn programı ile hesaplanmıştır. SK-1 and PARP kesimi western blot ile belirlenmiştir. Midostaurin, SKI II ve kombinasyonlarının hücre döngüsü dağılımları PI boyaması, apoptoz ise aneksin V/PI ikili boyaması yapılarak akım sitometresi ile saptanmıştır.

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Bulgular: Midostaurin SK-1 ifadesini azaltmıştır. Midostaurin, SKI II ve belirli konsantrasyonlardaki kombinasyonlar hücre canlılığını doza bağlı olarak azaltmıştır. Midostaurinin SKI II ile kombinasyonu aditif etki göstermiştir. Kombinasyon muamelesi fosfatidilserinin dış membran yüzeyine translokasyonunu ve PARP aktifleşmesi ile apoptozu tetiklerken aynı zamanda nekroza ve hücre döngüsünün G0/G1 ve S fazlarında tutulumlara neden olmuştur.
Sonuç: Klinik öncesi detaylı calışmalar ile desteklendikten sonra sfingozin kinaz-1'in FLT3 ile birlikte hedeflenmesi yabanıl tip FLT3 ifadesine sahip AML'de midostaurinin klinikte görülen sınırlı etkisinin arttırılması açısından yeni bir yaklaşım olabilecektir.

Anahtar Kelimeler: apoptoz; FLT3 yabanıl tip AML; midostaurin; sfingozin kinaz; sfingozin kinaz inhibitörü.

Introduction

Acute myeloid leukemia (AML) is a highly aggressive and heterogeneous disorder mostly observed in individuals older than 60 years old who possess abnormally proliferating myeloblasts in their blood and bone marrow [1]. Prognostic AML subtypes have been defined based on the molecular identification of chromosomal and genetic mutations including the Fms-like tyrosine kinase 3 (FLT3) abnormalities [2].

FLT3 gene encodes a type III receptor tyrosine kinase expressed in hematopoietic stem cells and early progenitor cells during hematopoietic and immune system development, then its expression becomes very weak or completely absent in functionally differentiated cells [3, 4]. Binding of FLT3 ligand (FL) to FLT3 activates downstream RAS, PI3K/AKT and STAT5 signaling pathways leading to cell proliferation and suppression of apoptosis [4]. The FLT3 is overexpressed and mutated in majority of AML cases, displaying its importance as an attractive therapeutic target. Two most common FLT3 mutations include tyrosine kinase domain (TKD) mutations and internal tandem duplication (ITD) of its juxtamembrane domain with prognostic and therapeutic obstacles [5, 6]. These mutations result in constitutive activation of FLT3 kinase and its downstream proliferative signaling cascades, which inhibit apoptosis and promote further proliferation [4, 5]. Discovering the molecular roles of FLT3 in disease formation has been considered as a milestone in the treatment of FLT3 positive AML through the development of FLT3 inhibitors, which are grouped as first (including midostaurin and sorafenib) and next generation inhibitors (including crenolanib and gilteritinib) based on their specificity and potency [6].

Midostaurin (Rydapt, Novartis Pharmaceuticals Corp.) is the first multi-kinase inhibitor approved by the US Food and Drug Administration (FDA) in 2017 for newly diagnosed FLT3-mutated AML in combination with daunorubicin and cytarabine induction and consolidation based on the results of RATIFY trial [7]. Additionally, some clinical results showed midostaurin transient effectiveness in wild-type FLT3-expressing AML due to the presence of wild-type FLT3 overexpression in majority of AML patients (70–100%) and its multi-targeted nature in addition to FLT3 inhibition [8, 9]. However, pre-clinical investigation of midostaurin in FLT3 overexpressing AML remains very limited. These studies showed that there could be different non-FLT3 targets modulating midostaurin's efficacy in the clinic including SYK, KIT, ERK and MEK kinases [10, 11]. Therefore, identification of novel FLT3-independent targets such as sphingolipid metabolism enzymes could be contributed to understand how this broad-spectrum activity of midostaurin has been observed in FLT3 wild-type AML and could open the way of new combination strategies for the treatment.

Manipulating sphingolipid metabolism is considered as an emerging approach in cancer therapy, since they have significant roles in the regulation of cell division, cell growth, metastasis, apoptosis and therapy response in addition to being structural components of cellular membranes [12]. Sphingolipid family includes ceramide (Cer) as a central lipid, sphingosine-1-phosphate (S1P) and glucosylceramide (GC), which regulate initiation and progression of cancer [13, 14]. Among different members of sphingolipid metabolism, Cer produced through *de novo* synthesis pathway or salvage pathway is known as a second messenger in cell death [15]. On the other hand, Cer could be converted into S1P by sphingosine kinase (SK-1 or SK-2), which is a pro-survival signal [16]. Therefore, the strict balance between apoptotic Cer and anti-apoptotic sphingolipids determines the cell fate called as "sphingolipid rheostat" [17]. The metabolizing enzymes have been considered as potential biomarkers and chemotherapeutic targets in various malignancies [12]. In leukemia cells, SK-1 was upregulated and its synergistic inhibition together with sirtuin 1 was shown to suppress leukemia growth [18]. Large granular lymphocyte (LGL) leukaemia cells have higher expression of SK, which were inhibited using either genetic or pharmacological approaches and resulted in suppression of leukemia proliferation through proteosomal degradation of anti-apoptotic Mcl-2 [19]. SK-1 was overexpressed and activated in AML patient blasts and its inhibition resulted in caspase-dependent cell death in AML cell lines and decreased tumor burden and prolonged survival in patient-derived xenograft model

[20]. Therefore, it could be important to elucidate the potential roles of sphingolipid metabolizing enzymes in pathogenesis and response to midostaurin treatment in FLT3 wild-type AML.

In this study, we aimed to explore activity of midostaurin alone and in combination with sphingosine kinase inhibitor (SKI II) in wild-type FLT3 overexpressing AML for the first time, to better understand and explain the observed clinical efficacy and suggest a possible combination approach to increase its effectiveness in the clinic.

Materials and methods

Chemicals

MTT and midostaurin were purchased from Sigma-Aldrich (USA). SKI II was obtained from Cayman Chemicals (Ann Arbor, MI, USA). 10 mM stock solutions were prepared in DMSO. The final concentration of DMSO did not exceed more than 0.01% in culture. Penicillin-streptomycin, RPMI 1640, and fetal bovine serum were obtained from Invitrogen (Paisley, UK). The non-treated cells were used as control in experimental setups.

Cell lines and culture conditions

Human THP1 FLT3 overexpressing AML cells obtained from German Collection of Microorganisms and Cell Cultures (Germany, DSMZ). The Cells were cultured in RPMI-1640 growth medium (with L-glutamine) including 10–20% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37 °C in a 5% CO_2 incubator. The conducted research is not related to either human or animal use.

MTT cell proliferation assay

Concentration-dependent anti-proliferative effects of midostaurin (100–1,000 nM) and SKI II (1–80 μM) on THP1 cells were determined by MTT cell proliferation assay [21, 22]. THP1 cells were seeded in 96 well plates at density of 1×10^4 cells/well and incubated for 48 h. 20 μL MTT solution (5 mg/mL, Sigma Aldrich) was added to each well after incubation to form formazan crystals dissolved in 100 μL DMSO. Then, absorbance values were measured at 570 nm by a spectrophotometer. Based on the cell proliferation graphs, IC_{50} values (concentration inhibiting cell growth by 50%) for midostaurin and SKI II were calculated by linear regression analysis using GraphPad software (San Diego, CA). Relative cell viability was calculated as the ratio of the absorbance at 570 nm of each treatment group against those of the corresponding untreated control group.

Calculation of combination indexes (CIs)

Increasing concentrations of midostaurin were combined with increasing concentrations of SKI II at a fixed molar ratio for 48 h. Then, the therapeutic effect of co-treatments was assessed by MTT assay. Combined-effects analyses, based on the method of Chou and Talalay, were performed to establish whether combinations result in synergism, additivity or antagonism using CalcuSyn software (Biosoft, Cambridge, United Kingdom [23]. A CI of <1, 1.0–1.1, or >1.1 is indicative of synergistic, additive/nearly additive, or antagonistic effects, respectively [24].

Annexin-V FITC/PI double staining by flow cytometry

Annexin V-FITC apoptosis detection kit (BioVision, USA) was used to examine apoptotic effects of midostaurin (400 and 800 nM) and SKI II (20 and 40 μM) alone and in combination (400 and 800 nM midostaurin with 20 and 40 μM SKI II, respectively) according to manufacturer's instructions. Briefly, 7.5×10^5/2 mL cells seeded into each well of 6-well plate were exposed to indicated treatments for 48 h. Then, the cells were collected, washed twice with cold PBS. 2 μL of FITC Annexin V and 2 μL of propidium iodide (PI) were added to the cells and incubated for 15 min [21]. The percentage of early and late apoptotic cells were determined by flow cytometry using a BD FACSCalibur flow cytometer (BD Biosciences) within 1 h. The results were analyzed using BD FACSDiva™ (BD Biosciences).

Cycle analysis

The cells were seeded into a 6 well plate at a density of 7.5×10^5 cells/well and treated with midostaurin (400 and 800 nM) and SKI II (20 and 40 μM) alone and in combination (400 and 800 nM midostaurin with 20 and 40 μM SKI II, respectively) for 48. Then, the cells were fixed with 3 mL cold ethanol for at least 24 h in –20 °C. Samples were centrifuged at 260 g for 10 min and homogenized in 5 mL cold PBS and centrifuged. 1 mL PBS-Triton X100 and 100 μL RNase-A (200 μg/mL, Sigma Aldrich) were added and incubated at 37 °C for 30 min. Then, 100 μL PI (1 mg/mL, Sigma Aldrich) was added and incubated at room temperature for 10–15 min [21]. Cell cycle analysis was carried out by BD FACSCalibur flow cytometer (BD Biosciences) with BD FACSDiva™ (BD Biosciences) software.

Protein extraction and western blot

5×10^6 cells were treated with midostaurin (200 and 400 nM for SK-1 expression) alone or in combination with SKI II (400 nM midostaurin with 20 μM SKI II for PARP cleavage) for 48 h to detect the changes in the expression of SK-1 and PARP by western blot, respectively. Cells were lysed in RIPA buffer (Sigma-Aldrich, USA) and protein concentrations were measured using RC DC™ Protein Assay Kit (Bio-Rad, USA). 30 μg/well total protein was separated by 8–15% SDS-PAGE and transferred to PVDF membranes. The membranes were blotted with primary antibodies for SK-1 (1:3,000, Cell Signaling, USA), PARP (1:3,000, Cell Signaling, USA) and Beta Actin (1:3,000, Cell Signaling, USA) overnight at +4 °C and conjugated with appropriate secondary antibodies (1:10,000, Jackson Immuno Research, USA). The membranes were visualized with Pierce™ ECL Western Blotting Substrate kit (Thermo Scientific™, USA). Densitometric analysis of immunoreactive bands were carried out by using the imaging software (Bio-Rad, ChemiDoc, Image LabTM 3.0).

Statistical analysis

The experiments were performed in at least two independent setups and the results are given as mean ± standard deviation (SD).

Statistical analysis was performed using GraphPad Prism 6.0. One-way ANOVA was used for the analysis followed by Dunnentts' or Tukey's multiple comparisons test. $p<0.05$ was considered as statistically significant for all data.

Results

Sphingosine kinase-1 is downregulated in wild-type FLT3 overexpressing AML cells after midostaurin treatment

To figure out whether multi-kinase inhibitor midostaurin has a direct effect on SK-1, which produces anti-apoptotic S1P, alterations in the protein level of SK-1 in response to 48 h 200 and 400 nM midostaurin exposure (concentrations lower than IC_{50} values of midostaurin selected to obtain appropriate amount of proteins without causing massive cell death) [10] were analyzed. Treatment of THP1 cells with 400 nM midostaurin significantly decreased SK-1 protein expression (approximately 30% decrease) compared with the untreated control group (Figure 1). Therefore, the results demonstrated for the first time that SK-1 could be a functional enzyme in FLT3 wild-type leukemia cells and may be considered as an important target for midostaurin treatment.

In vitro combined anti-leukemic effects of midostaurin and SKI II

To assess if combining midostaurin and SKI II results in synergistic cytotoxicity, THP1 cells were exposed to various concentrations of midostaurin, SKI II or combinations. After 48 h of exposure, cell viability was measured by MTT assay. As shown in Figure 2A, B, treatment with midostaurin or SKI II alone inhibited the viability of THP1 cells in a dose-dependent manner. IC_{50} values for midostaurin and SKI II were approximately 916 nM and 42 μM, respectively. To explore combined drug effects, we exposed THP1 cells to the constant molar ratio of midostaurin and SKI II by using concentrations below their IC_{50} values for 48 h. Midostaurin and SKI II in combination further suppressed FLT3 overexpressing AML cell viability in a dose-dependent manner, compared with the control (Figure 2C). Only 800 nM midostaurin plus 40 μM SKI II combination decreased cell viability compared to midostaurin alone (Figure 2C). Combination indexes (CI) were also calculated for three combinations used in cell viability assay (Figure 2D). The combination of 400 nM midostaurin and 20 μM SKI II was additive (CI:1) while other two combinations were slightly antagonistic (CI: 1.2).

Midostaurin in combination with SKI II induces G0/G1 and S phase cell cycle arrest

The combined effect of midostaurin and SKI II on THP1 cell cycle progression was investigated. Midostaurin treatment alone induced cell cycle arrest at the G2/M phase (36.45 and 9.5%, respectively), whereas SKI II alone induced cell cycle arrest at S phase (43.5 and 26.7%, respectively) as compared to control (Figure 3A, B). Combination of 800 nM midostaurin with 40 μM SKI II significantly arrested the cell cycle at the S phase (46.95 and 26.7%) as compared to control. Same combination also resulted in G0/G1 arrest as compared to midostaurin alone (44.65 and 26.2%, respectively). These data indicated that combined agents showed a more significant suppressive effect on cell cycle progression than each agent alone.

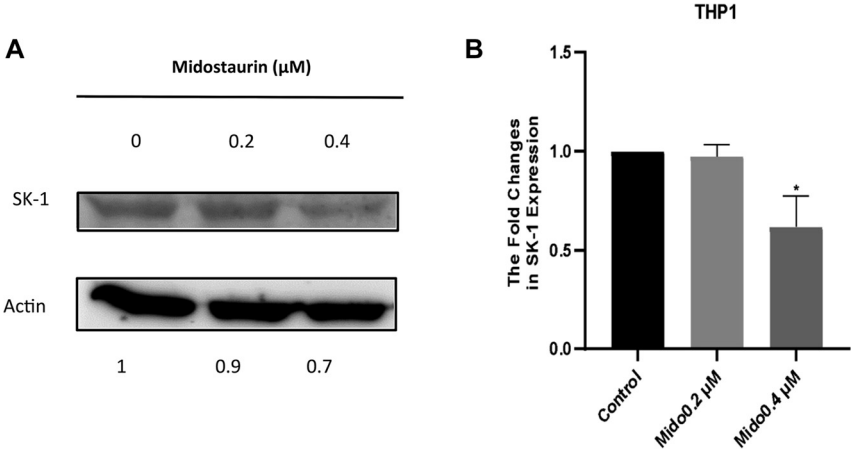


Figure 1: SK-1 expression in midostaurin-treated THP1 (A) and (B) cells for 48 h. Beta-actin was used as a loading control. The results from at least 2 different experimental sets were given as mean ± SD
*$p<0.05$ vs. control.

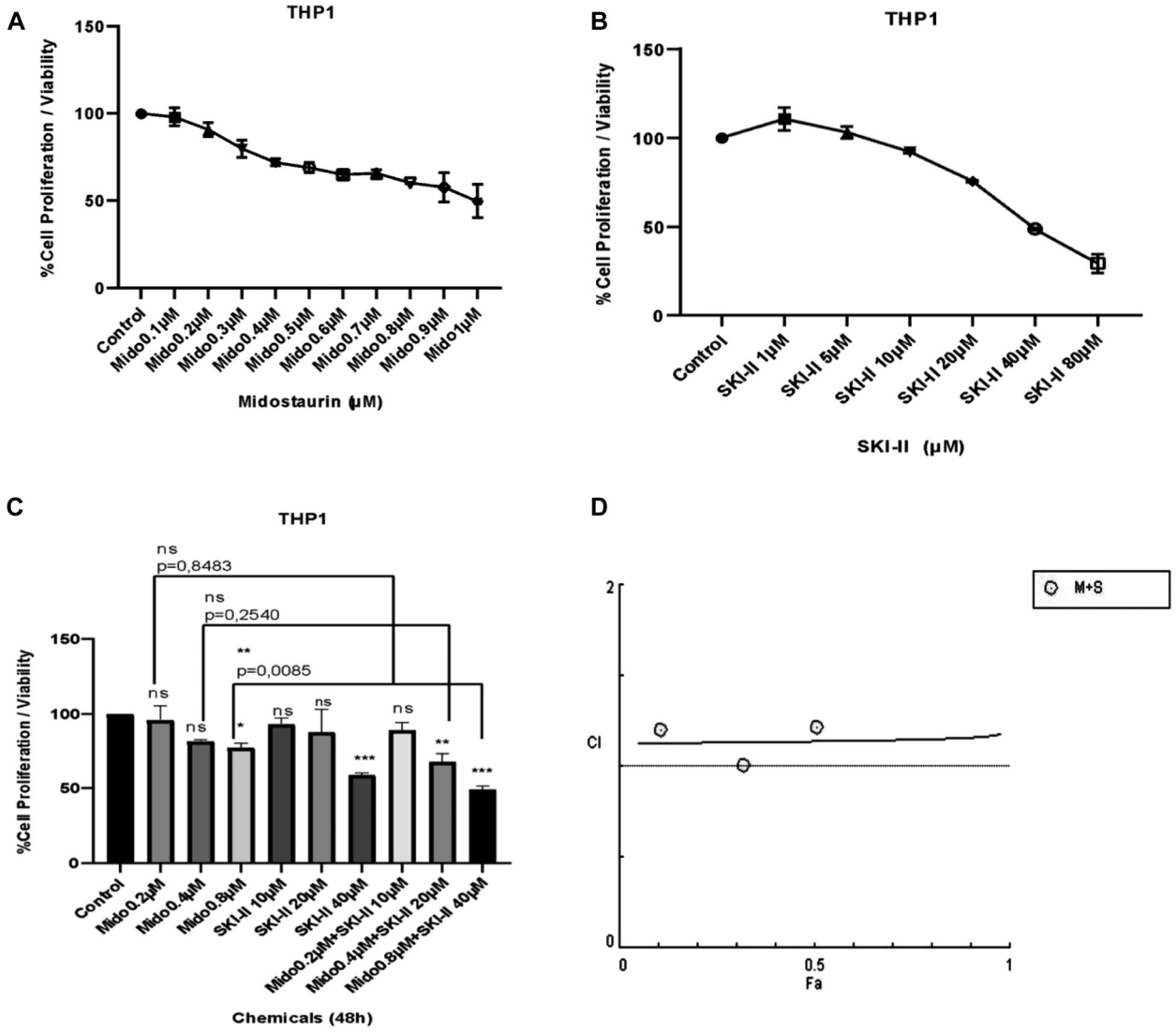


Figure 2: Cytotoxic effect of midostaurin (A), SKI II (B) and combinations (C) in THP1 cells at 48 h. Data are shown as the mean ± SD of at least two independent experiments. Calcusyn analysis of midostaurin plus SKI II (D).*p<0.05, **p<0.005, ***p<0.0001 vs. control.

Midostaurin plus SKI II induces apoptosis via PARP cleavage

It was explored whether midostaurin in combination with SKI II may trigger a synergistic effect on THP1 cell apoptosis. Midostaurin alone did not trigger significant apoptosis compared to the control. SKI II individually induced slight and moderate apoptosis, compared with the control group (5.3 and 6.7 vs. 3.3%, respectively) (Figure 4A, B). On the other hand, all combinations of midostaurin with SKI II triggered apoptosis (9.5 and 9.1 vs. 3.3%, respectively), compared to the control and midostaurin alone (9.5 and 9.1 vs. 4.15 and 3.75%, respectively). 400 nM midostaurin with 20 µM SKI II also resulted in PARP activation as compared to control and midostaurin alone (Figure 4C). In summary, these results proposed that midostaurin and SKI II combination decreased cell proliferation at certain concentrations via induction of apoptosis.

Discussion

As a first FDA approved FLT3 inhibitor, midostaurin, has provided improved clinical outcomes in FLT3-mutant AML in combination therapies with increased overall survival (OS) [25]. Although midostaurin treatment resulted in complete remission (74% of registered patients) with almost similar OS rates with FLT3-mutant AML patients [9]

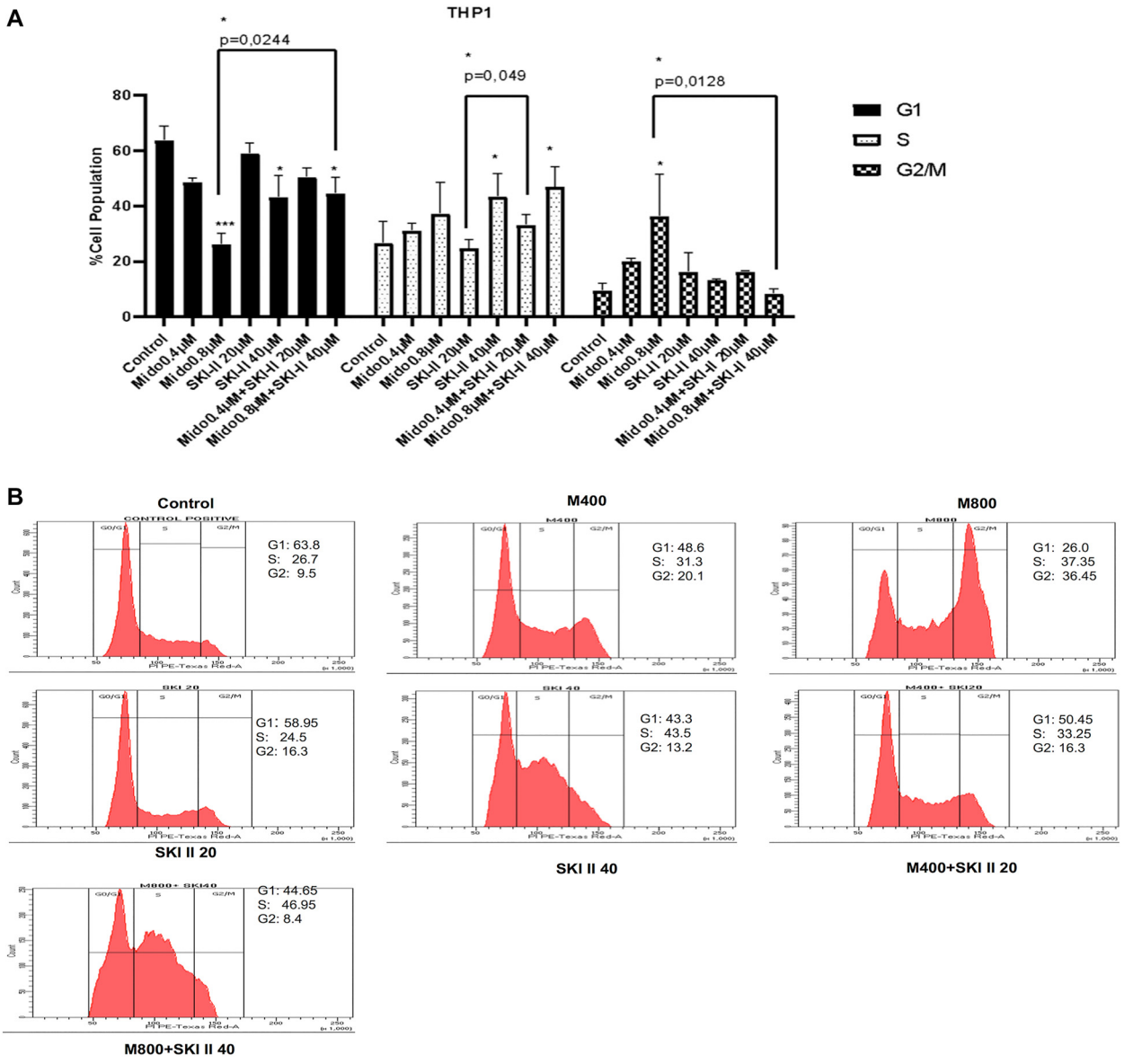


Figure 3: Cell cycle profiles of THP1 cells after midostaurin, SKI II and combination treatments for 48 h. Data are shown as the means ± SD of at least two independent experiments. *p<0.05, ***p<0.0001 vs. control.

and its administration as a combination with chemotherapy in newly diagnosed FLT3 wild-type AML showed promising results [26], its benefit still seems to be underestimated. There are limited studies explaining the molecular background of observed efficacy in midostaurin treated FLT3 wild-type AML patients, which suggested the modulation of FLT3-independent signaling pathways or molecules [10, 11]. Hence, in this study, we hypothesized that wide-spread effect of midostaurin could be due to non-specific inhibition of other kinases, such as SK-1 and targeting both FLT3 and SK-1 could be a promising combinational therapeutic strategy to benefit FLT3 wild-type AML patients more broadly.

SK-1 catalyzes the coversion of Cer into S1P, which oppose each other functions as apoptotic and anti-apoptotic lipids, respectively. Cer accumulation is one of mechanisms of chemotherapeutic drugs to induce apoptosis in cancers whilst S1P accumulation associates with a decrease in therapeutic efficacy, called "sphingolipid rheostat" [15, 17].

The present study suggested that the less sensitive behavior of FLT3 wild-type AML to midostaurin (mainly responding to higher submolar concentrations indicated in this study as compared to FLT3-mutated AML) [10] could be related to SK-1 expression in the cells. SK-1 protein expression was found to be decreased after midostaurin

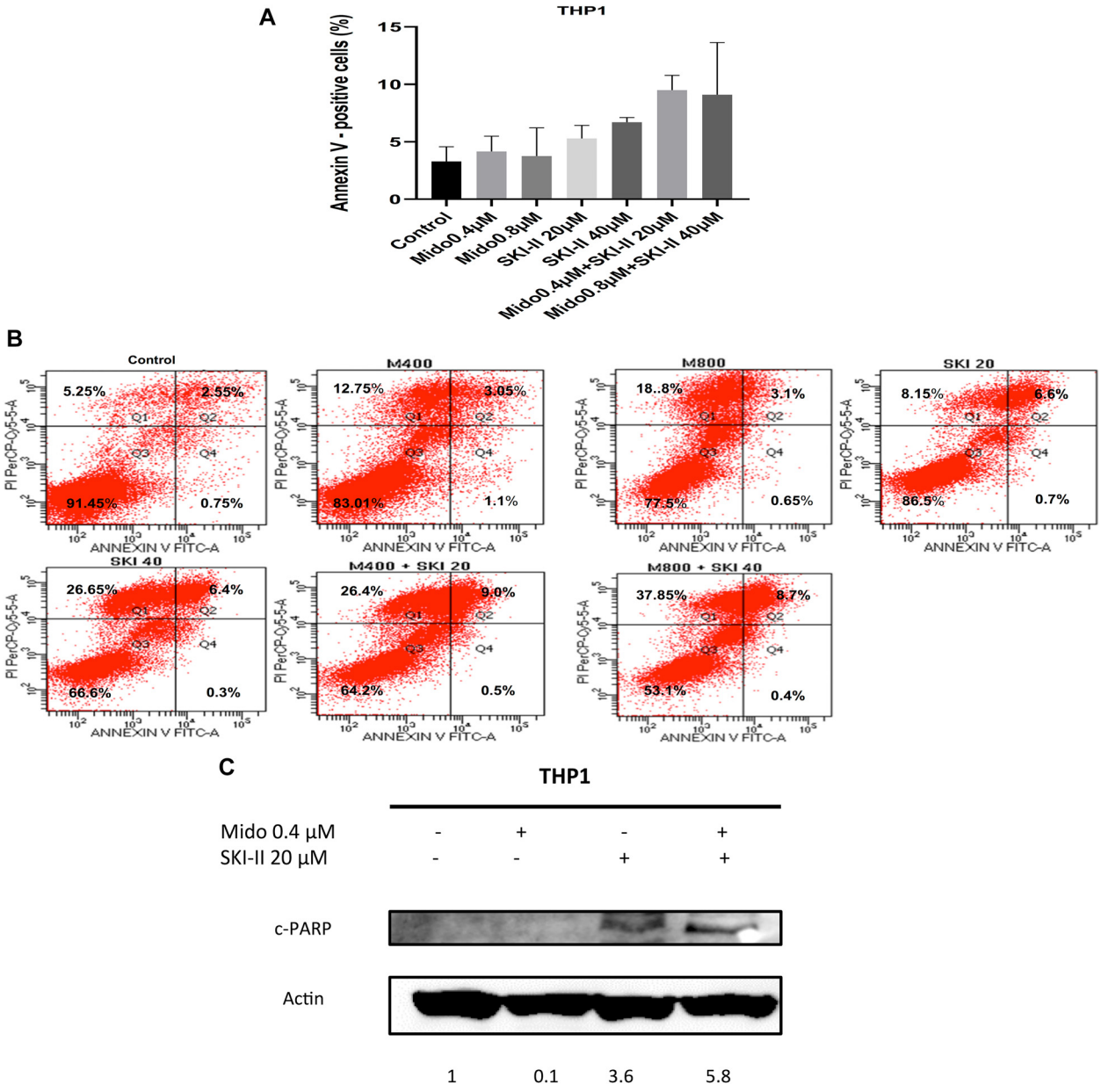


Figure 4: Apoptotic effects of midostaurin, SKI II and combinations on THP1 cells. The results derived from the means of at least two independent experiments are represented as mean ± SD. The percentage of annexinv-positive/PI negative early apoptotic population and annexinv-positive/PI positive late apoptotic population were calculated and normalized to untreated control or each agent alone (A) and (B). PARP cleavage in response to midostaurin, SKI II and the combination (C).

treatment as compared to untreated cells for the first time (Figure 1), suggesting that SK-1 might be a direct target of midostaurin and have a role in FLT3 wild-type AML pathogenesis. This data is compatible with scarce studies in different subsets of AML other than FLT3 positive AML including B-lineage acute lymphoblastic leukemia (ALL) and diffuse large B cell lymphoma [20, 27, 28] in which SK-1 was defined as a target for therapy.

Based on this data, midostaurin was combined with SK-1 inhibitor to observe whether there is a synergistic antiproliferative effect on THP1 cells. In Figure 1C, even though 400 and 800 nM midostaurin in combination with 20 and 40 μM SKI II, respectively inhibited cell viability as compared to control, only 400 nM midostaurin with 20 μM SKI II achieved additive effect. Cell viability and combination analyses are not always supported each other as

shown in different studies [29]. Therefore, 400 nM midostaurin with 20 μM SKI II could be suggested as an appropriate combination since it also increased cell death (Figure 4B) and PARP activation (Figure 4C). Before suggesting exact combination, it is still needed to test this combination in *in vivo* models which might show different responses than *in vitro* studies due to the presence of different factors such as pharmacodynamic and pharmacokinetic behaviours affecting drug-drug interactions [30].

The further data displayed that midostaurin's cytotoxicity in THP1 cells was potentiated at certain combinations via arresting cell cycle at G0/G1 and S phases and inducing apoptosis (Figures 2–4). Targeting SK-1 by using genetic or inhibitor-based approaches in hematological malignancies inhibited cell proliferation through different mechanisms such as externalization of phosphatidylserine (PS), caspase and PARP activation and cell cycle arrest at different phases [18, 27, 31], which further supported the results obtained from the treatment of THP1 cells only with SKI II (S phase arrest and externalization of PS and PARP cleavage, Figures 3, 4) in this study. For instance, SKI II arrested cell cycle at G0/G1 phase in chronic myeloid leukemia cells (CML) [18] and induced apoptosis in HL60 and U927 AML cells in a caspase-dependent manner [32]. Time-dependent PARP cleavage and PS relocalisation observed after treatment with SK inhibitor in T-ALL [33]. On the other hand, apoptosis induction was not significant after midostaurin treatment as shown in flow cytometry and PARP cleavage data (Figure 4), which was in accordance with the literature [34] in which midostaurin treatment did not provoke apoptosis in FLT3 wild-type AML cells. Midostaurin alone led to G2/M arrest in THP1 cells compared to control (Figure 3), which was supported by the study in which its different effects on cell cycle progression was compared with FLT3 mutant AML [34]. In addition to apoptosis, midostaurin and SKI II caused necrotic cell death in THP1 cells (Figure 4B). SK inhibitors including PF-543 and SKI II were shown to induce both necrosis and apoptosis in head and squamous and merkel cell carcinoma cells as a major cell death mechanism [35, 36]. Co-treatments also caused increased necrotic cell death based on the results of annexin- V/PI staining (Figure 4B), which could be derived from the necrotic effect of each agent alone. This data needs to be further clarified mechanistically whether detected cell death could be a programmed version of necrosis called necroptosis by using necrostatin-1 (inhibitor of necrosis). It is well defined that necroptosis, showing similarities with both necrosis and apoptosis, could also be an alternative mode of cell death caused by chemotherapeutics or agents [37, 38].

Midostaurin has demonstrated anti-cancer activity in combination with agents targeting pro-survival pathways or molecules mainly in FLT3-ITD AML, to lesser extent, in FLT3 wild-type AML. Co-targeting of FLT3 with midostaurin and MEK showed synergism in FLT3 wild-type AML [11]. OCI-AML2 FLT3 wild-type AML cells responded synergistically to midostaurin plus Mcl-1 inhibitor S63845 [10]. However, there are limited studies demonstrating the contribution of sphingolipid metabolism to midostaurin's effect. In head and squamous cancer, liposomal Cer administration increased sensitivity to midostaurin [39]. Inhibition of glucosylceramide synthase in the presence of midostaurin was found to enhance midostaurin's cytotoxicity in FLT3 overexpressing cells [40].

In conclusion, the data obtained in this study could reveal the role of SK-1 inhibition to increase midostaurin's activity in FLT3 wild-type AML for the first time through inducing cell cycle arrest, apoptotic and necrotic cell death. SK-1 inhibition could be considered a new integrative approach in combination with midostaurin to increase the usage of midostaurin more broadly after performing further mechanistic pre-clinical *in vitro* and *in vivo* studies and clarifying the exact mode of cell death mechanism based on the data obtained from this study.

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