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Cytotoxic and Cytostatic Effects of Targeting mTOR and Hedgehog Pathways in Acute Myeloid Leukemia

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ABSTRACT

Objectives: Acute myeloid leukemia (AML) is a highly aggressive heterogeneous hematopoietic malignancy characterized by a rapid and abnormal proliferation of immature myeloid leukemia cells in the bone marrow and peripheral blood. Aberrant alterations in signal transduction pathways are strongly associated with the progression of AML. This study aimed to investigate cell viability and the cell cycle in AML cells by targeting the Hedgehog and mTOR signaling pathways with rapamycin and GANT61.

Materials and Method: The antiproliferative effect of rapamycin and GANT61 was assessed by the MTT cell viability assay in two AML cell lines: CMK and MOLM-13. The effect of the inhibitors on cell-cycle distribution was determined using propidium iodide staining and measured with flow cytometry.

Results: Rapamycin, an mTOR inhibitor, and GANT61, a Gli-1 inhibitor, decreased the cell proliferation of CMK and MOLM-13 cells. The IC20 values, which is the drug concentration that inhibits cell growth by 20%, were combined and administered to the cells. The results show the drugs to have a combinatorial inhibitory effect on CMK cells but not on MOLM-13 cells. In addition, the combination of drugs arrested the cells during the G0/G1 phase.

Conclusion: This study suggests a novel combination therapy approach for AML via mTOR and Hedgehog signaling pathway inhibition using rapamycin and GANT61, respectively. It also suggest further studies be performed to reveal the mechanism of action.

Keywords: Hedgehog, mTOR, leukemia, combination therapy, cell cycle

INTRODUCTION

Acute myeloid leukemia (AML) is a highly aggressive heterogeneous hematopoietic malignancy characterized by a rapid proliferation of immature myeloid leukemia cells in the bone marrow and peripheral blood (1-3). According to Saultz and Garzon (3), AML arises from the genetic abnormalities of leukemia cells that accumulate with age. AML has an effect on marrow in the majority of patients, with 20% of the affected bone marrow cells being immature or undifferentiated leukemia blasts (2).

Alterined proliferation and survival mechanisms are common deregulations in AML cells. These mechanisms are under the control of signal transduction pathways, and some of the signal transduction pathways affected in AML cells are receptor tyrosine kinases such as FLT3 and KIT and non-receptor tyrosine kinases such as RAS family members, RAF/MEK/ERK, and PI3K/AKT, as well as the Hedgehog (HH) and mTOR signaling pathways. These pathways are mostly affected by means of gain-of-function mutations in upstream elements that alter the activation mechanism. Constitutive activation of these pathways leads to the survival and proliferation of hematopoietic progenitor cells (4). Although AML is the second most commonly seen leukemia in children, the survival rate of AML patients has not improved due to the heterogeneity of the disease. The

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need exists to target specific signaling pathways to accomplish an efficient treatment approach toward AML.

The HH signaling pathway is conserved and functions in developmental processes such as regeneration, differentiation, and proliferation (5). Even though the HH signaling pathway is conserved, its function varies in different species. For adult humans, the HH signaling pathway has responsibilities in tissue repair, stem cell renewal, and oncogenesis (6). The role of the HH signaling pathway regarding stem cell renewal in both somatic stem cells and pluripotent cells can be correlated with cancer stem cell activity when alterations occur in the signaling pathway and its activity (7). HH signaling is aberrantly expressed in AML cells, suggesting a link between poor prognosis and drug resistance (8). The HH signaling pathway includes glioma-associated oncogene (Gli) proteins (Gli1, Gli2, and Gli3), which are transcription factors. GANT61 is a selective inhibitor of Gli-1 gene transactivation. *In vitro* studies have been conducted for many types of cancer, including neuroblastoma, rhabdomyosarcoma, colon, lung, pancreatic, leukemia, cervix, gastric, skin, and head-neck (9-11).

The mammalian target of rapamycin, also known as mTOR, is the pathway responsible for the regulation of growth and cellular metabolism in eukaryotic cells by regulating cell proliferation, autophagy, and apoptosis through multiple signaling pathways. mTOR is a serine/threonine protein kinase that forms two distinct catalytic subunits of the mTOR complexes, mTORC1 and mTORC2 (12, 13). PI3K/AKT and mTOR signaling have been documented to support the proliferation and survival of AML cells (14). mTOR activation is mostly observed in AML blasts, but the precise function and the downstream targets of mTOR in the AML are poorly understood. Meanwhile, rapamycin, which is an mTOR inhibitor, has been proven as a promising class of agents for treatment of malignant blood cancer, either alone or in combination with other treatments (15).

The inhibition of a single pathway, especially the mTOR or HH pathway, has been targeted by numerous researchers and reviewed previously. However, targeting a single pathway related to AML is an insufficient approach due to the presence of simultaneous alterations of multiple signaling transduction mechanisms. Therefore, the development of a combination therapy targeting the mTOR and HH pathways is needed in order to provide an efficient treatment option for AML patients. This study aimed to target the mTOR and HH pathways in AML patients to understand the crosstalk between these two pathways in order to find a possible therapeutic approach. This study hypothesizes that inhibiting the mTOR and HH signaling pathways using rapamycin and GANT61 is a combination that could be a therapeutic approach for AML. Inhibiting these pathways has been investigated by numerous studies due to their high potency as therapeutic targets. However, no reports are found concerning the effect of the drugs used in the current study on both of these pathways in AML cells.

MATERIALS AND METHODS

Chemicals

Rapamycin and GANT61 were purchased from Biovision and Sigma, respectively. Five mM stock drug solutions were prepared in dimethylsulfoxide (DMSO) in accordance with the recommendations and stored at -20°C. The RPMI 1640 and penicillin/streptomycin were obtained from Euro Clone. The heat-inactivated fetal bovine serum (FBS) was obtained from Biological Industries. RNAse and propidium iodide (PI) were purchased from Sigma and used in the cell cycle assay.

Cell Culture and Maintenance

The AML cell lines CMK and MOLM-13 were obtained from the German National Resource Center for Biological Material (DSMZ). CMK was sourced from the AML M7 cell line of Down syndrome patients (DS-AMKL), and MOLM-13 was originated from a FLT3 mutation. The cells were cultured in the RPMI growth medium supplemented with 20% FBS and 1% penicillin/streptomycin at 37°C and 5% CO_2.

Cell Viability Assay

The cell viability assay was performed to determine cell proliferation using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) as previously described (16). In brief, 96 well plates were seeded with $1x10^4$ cells/wells treated with increasing concentrations of inhibitors. After 48 hours, the MTT solution was added and incubated for 2 h. Following the incubation time, the plates were centrifuged for 10 min. The formazan crystals that formed were dissolved using DMSO. The plates were left for 15 min on a rotator. Afterward, the absorbance was measured at 570 nm using the microplate reader (Varioskan™ LUX multimode, Thermo Scientific).

Cell Cycle Assay

$1x10^6$ cells were plated into the cells and treated with rapamycin and GANT61 for 48 h. After collecting and centrifuging the cells at 260g for 10 min at 4°C, the supernatant was collected and the cells were washed twice in cold PBS. Following the removal of the supernatant, the pellet was dissolved with a mixture of 1 mL cold PBS and 4 mL ethanol (70%) and incubated at -20°C overnight for fixation. After incubation, the supernatant was removed by centrifugation, and the cell pellet was washed in PBS. The PBS/Triton X-100 was added next, followed by the addition of 100 µL of RNase-A, which were then incubated at 37°C for 30 minutes. Lastly, 100 µL of PI was added, incubated for 15 minutes, and analyzed using flow cytometry.

Statistical Analyses

Analysis of the viability data was done with the GraphPad PRISM (version 8.0; GraphPad Software Inc.). The Dunnett test and one-way analysis of variance (ANOVA) were performed for the statistical analysis, which showed $p = 0.0001$ for all results, with $p = 0.0005$ for the MOLM-13 combination cell viability results. Assays were repeated three times independently from one another.

Table 1. Combination index (CI) was calculated and isobolograms determined using the program CompuSyn.

CMK			MOLM-13		
GANT61	**RAPAMYCIN**	**GANT61+RAPA**	**GANT61**	**RAPAMYCIN**	**GANT61+RAPA**
Dose	Dose	CI Value	Dose	Dose	CI Value
5.2	0.1	0.56278	1.74	0.1	0.23773

The effects of the drug combination used in this study were evaluated using the CI based on Chou-Talalay's multidrug effect equation, with a CI of < 1, =1, or > 1 being respectively indicative of synergistic, additive, or antagonistic effects [17].

RESULTS

1. GANT61 and Rapamycin Decrease Cell Proliferation in AML Cell Lines

The study has assessed the cytotoxic effects of the HH inhibitor GANT61 and mTOR inhibitor rapamycin on the CMK and MOLM-13 AML cells. Increasing concentrations of the drugs were administered to the cells over 48 h, with the cell proliferations of both the CMK and MOLM-13 cell lines decreasing as the dosage increased. When GANT61 was administered between 5-100 μM, the IC20 values were determined as 5.2 μM for the CMK and 1.74 μM for the MOLM-13 cells. This result indicates GANT61 to be effective at inhibiting cancer cell survival by blocking Gli-1 activity (Figure 1a). Rapamycin also decreased cell viability when applied at 0.1-10 nM levels. However, the decrease was capped at 54.98% for a drug concentration of 0.5 nM or higher in both the CMK and MOLM-13 cell lines. After the rapamycin treatment, the IC20 value was calculated as 0.1 nM for both CMK and MOLM-13 cells (Figure 1b).

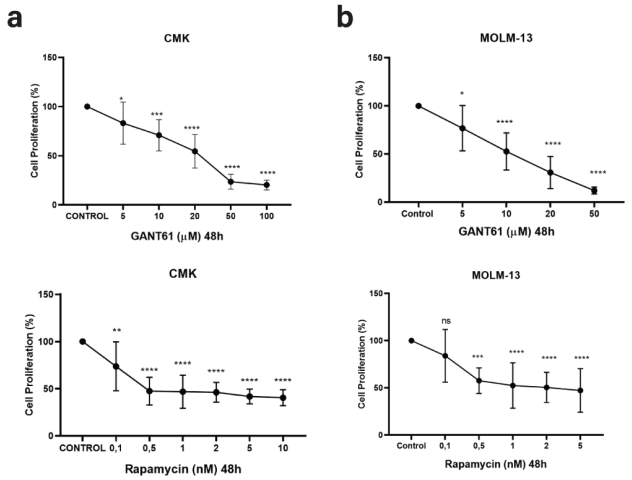

Figure 1. The effect of GANT61 and rapamycin on cell viability compared to the control for the (a) CMK and (b) MOLM-13 cells. These results represent data from samples in triplicates across three independent experiments ($n = 3$). * = $p < 0.05$; *** = $p < 0.001$; **** = $p < 0.0001$. *SD* = the mean value of the replicates; ns= Nonsignificant.

2. The Combined Cytotoxic Effects of GANT61 and Rapamycin on AML Cells

Next, the study evaluated the effect of drug combinations on AML cells. The cells were treated for 48 h with the IC20 concentration of rapamycin, which had been calculated as 0.1 nM for both cell lines, and the IC20 concentration of GANT61, which was detected as 5.2 μM and 1.74 μM for the respective CMK and MOLM-13 cells. Decreases in cell proliferation using the combination therapy were observed in the MOLM-13 cells by 46% and 38% for the respective untreated control and GANT61-only treatment. However, the cell viability following the combination treatment was surprisingly not significantly different when compared to the rapamycin-only treatment. The combination treatment for CMK cells decreased cell viability by 42%, 51%, and 10% when respectively compared to the untreated control, GANT61 treatment, and rapamycin-only treatment. Therefore, the results indicate the combination therapy to have reduced the cell proliferation levels more apparently in the CMK cells compared to the single treatments (Figure 2). However, when comparing the results regarding cell viability in terms of synergism, the combination of 5.2 μM and 1.74 μM GANT61 with 0.1 nM of rapamycin showed a synergistic effect (95% CI: 0.562-0.237) in the respective CMK and MOLM-13 cells (Table 1).

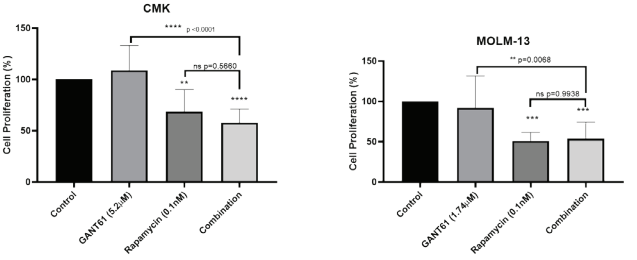

Figure 2. The cytotoxic effects of the different combinations of GANT61 and rapamycin on the CMK and MOLM-13 cells. These results represent the data from the triplicate samples across three independent experiments ($n = 3$).
** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$. *SD* = the mean value of replicates; ns= Nonsignificant.

Table 2. The cell cycle distribution and the percentage of the average cell population of CMK and MOLM-13 cells in G0, S, and G2/M phase.

	CMK cells			MOLM-13 cells		
	G0/G1	**S**	**G2/M**	**G0/G1**	**S**	**G2/M**
Untreated control	62.1	27.4	10.4	67.8	22.4	9.8
DMSO	63.2	26.5	10.3	66.4	23.85	9.5
GANT61 IC20	66.8	23.7	9.5	64.15	23.2	12.65
Rapamycin IC20	70.1	20	10	68.45	20.6	10.95
Combination	79.2	15	5.8	70	19.7	10.2

3. The Cytostatic Effect of the Combination Treatment on AML Cells

To enlighten the mechanism behind the decrease in cell proliferation, the cell cycle distribution was assessed in response to the drug combination treatment using the flow cytometric analysis. The results show the combination of GANT61 and rapamycin to have resulted in a 12% decrease in the S phase in the CMK cells when compared to the control. The combination treatment demonstrated a 17% increase in the G0/G1 phase, which shows the cells to have been arrested in this phase in response to the drug treatment (Figure 3a). In the MOLM-13 cells, similar effects were not observed as a result of the drug treatment. However, when compared to the CMK cells, a slight decrease was seen in the S phase, as well as a slight increase in the G0/G1 phase (Figure 3b). These results demonstrate the cytotoxic effect of the drug combination to perhaps have arisen from the cell cycle arrest (Table 2).

DISCUSSION

The therapeutic approach involves four types of standard treatments that are reported to have been used in chemotherapy, radiation therapy, and stem cell transplantation regarding AML. In addition to the standard treatments, new approaches are also being studied in clinics. Targeted therapy is one of the new therapies and uses drugs to attack cancer cells that cause less harm to normal cells. One subtype of targeted therapy is monoclonal antibody therapy, which uses antibodies for treatment to attach to and kill the cancer cells (18).

AML is a heterogeneous disease that makes targeting it with classical chemotherapeutic drugs difficult. Specific targeting of signaling pathways would be a more efficient way for treatment of this disease, and this study in particular has focused on simultaneously targeting the HH and mTOR signaling pathways with specific inhibitors due to the important role they represent in the progression of AML.

For instance, a correlation exists between the elevated expression of the HH-signaling protein Gli-1 and chemoresistance against therapeutic drugs and radiotherapy for AML (19, 20), with the proper HH signaling pathway activity being altered in AML cells. The induction of the signaling pathway in AML cells does not require HH to bind to PTCH1. Therefore, Gli-1 and SMO expression is persistent in AML cells. This persistence will increase the concentration of Gli-1 in AML cells (21). Subsequently, this increase in the Gli-1 alongside other elements of the signaling pathway result in AML becoming resistant to treatment. Queiroz et al. (21) demonstrated the HH pathway to be an important component of multidrug resistance in myeloid leukemia, as well as the need for it to be targeted. Inhibiting the HH signaling pathway has also been linked to the induction of apoptosis. Therefore, inhibiting this signaling pathway in AML cells is a viable and required strategy for treatment, in addition to administering HH inhibitors combined with chemotherapeutic agents targeting leukemic stem cells, as this pathway is involved in the maintenance and expansion of leukemic stem cells (22).

The second pathway targeted in the current study was mTOR signaling. mTORC1 functions as a cell growth and metabolism regulator and contains five components: mTOR, Raptor as the regulatory associated protein of mTOR, mLST8, PRAS40, and Deptor. mTORC2 has six components, some of which are common to mTOR, including mTOR again as well as Rictor, mSIN1, Proctor-1, mLST8, and Deptor again. In contrast to mTOR, the exact function of mTORC2 has not yet been revealed, but the known function of the complex is to control cell proliferation and cell survival (13, 23). By promoting anabolic processes and limiting catabolic processes, mTORC1 possesses a regulatory function in cell growth and proliferation (23). The base of knowledge about the function of mTORC1 involves bacterial macrolide rapamycin usage (24). Rapamycin is a macrocyclic antibiotic that was first discovered as an antifungal medication. However, its immunosuppressive effect is also considered useful as clinical medication (25). Rapamycin is an inhibitor that, by interacting with the FKBP12 protein and the FKBP12-

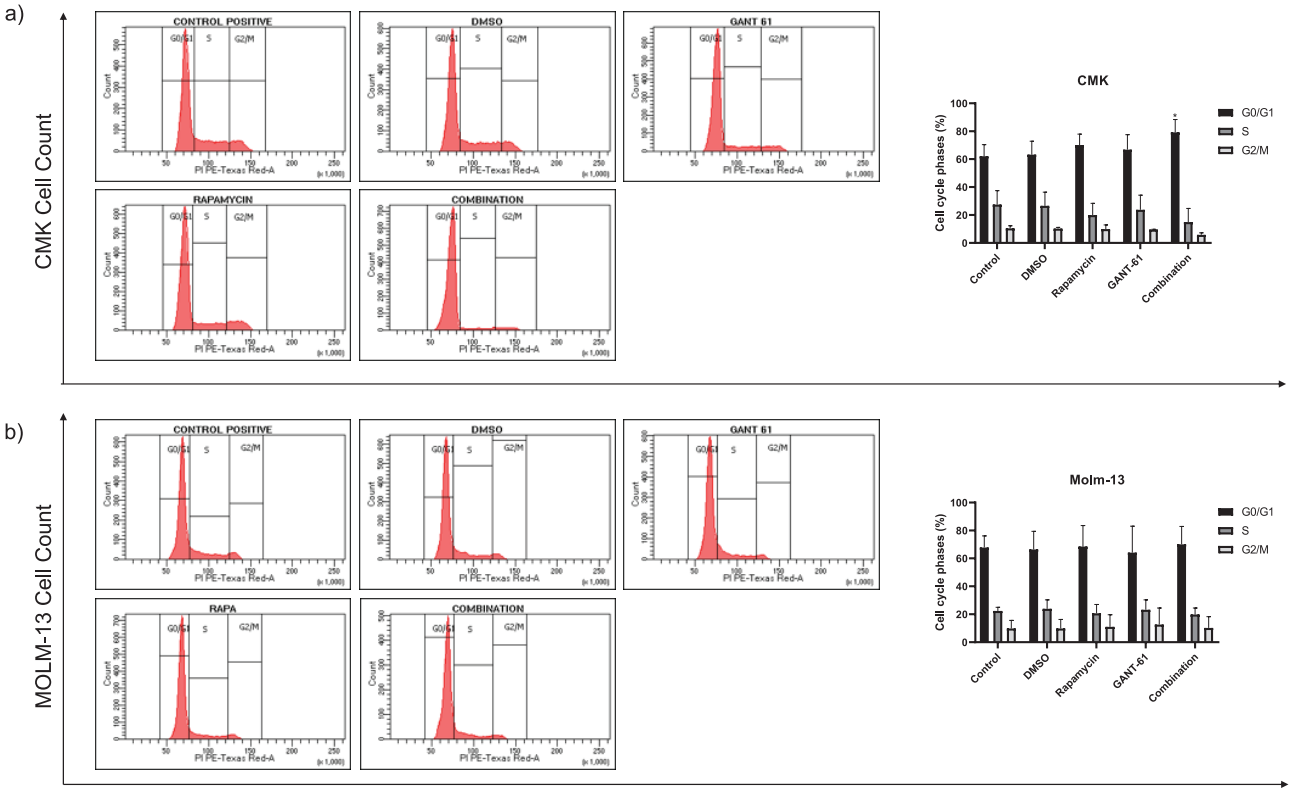

Figure 3. The effect of GANT61 and rapamycin alone or in combination on cell cycle progression of AML cells. CMK (n=3) and MOLM-13 (n=2). The representative histograms were shown. At least two independent experiments were performed, combined, and analyzed (* = p <0.05).

rapamycin binding domain of mTOR, inhibits the mTORC1 functions. The inhibition happens in the transition of mTOR and G1 to the S phase by forming an immunosuppressive complex. However, rapamycin is not able to physically contact or inhibit mTORC2. With respect to rapamycin sensitivity, mTORC1 and mTORC2 can respectively be defined as rapamycin-sensitive and rapamycin-insensitive. However, the paradigm might not be accurate when considering the cases of mTORC2 activity blockage by means of rapamycin or the mTORC1 resistance to rapamycin (23, 24). AML cells have an upregulated mTOR pathway that reprograms metabolic activities. For example, Akt is constitutively active and promotes glycolysis due to the upregulated mTOR pathway (2). In AML, the mTOR pathway becomes dysregulated once mutations occur in association with oncogene activation, oncogene amplification, or tumor suppressor gene inactivation. For instance, the deletion of PTEN activates the mTOR pathway by inducing p53 expression and leukemogenesis (1).

Alterations of key mTOR signaling genes have shown regulation of AML leukemia stem cells. An example of this is the Rheb1 expression, which is overexpressed in patients with AML and also associated with lower survival rates in comparison to patients with a lower expression of Rheb1. Rheb1 deletion also has revealed an induced effect of apoptosis and enhanced cell cycle arrest, leading to a prolonged lifetime in leukemia stem cells (1). Another study showed in their leukemia cell panel and pilot clinical study how rapamycin had demonstrated antileukemic effects (26). In addition, this aforementioned pathway implies constitutive activity in around 60% of the patients with AML, with constitutive activation causing a decrease in overall survival time (2). Therefore, inhibition of the mTOR pathway is crucial as a therapeutic approach for AML inhibition due to its vital functions such as proliferation and differentiation.

Together with these, the induced mTOR signaling pathway is seen to influence the HH signaling pathway via the phosphorylation of Gli-1 at Ser84. This phosphorylation ends up inhibiting SUFU binding, thereby inducing the HH pathway (27). This crosstalk between these two pathways has led this study to suggest that targeting them simultaneously would have a synergistic effect on AML cells due to the blockage of the signal that is triggered by both pathways. For this purpose, the study administered GANT61 as an inhibitor for the Gli-1 and Gli-2 proteins in the HH signaling pathway and rapamycin as an inhibitor of mTOR (9). GANT61 binds to Gli proteins inside the nucleus without targeting the direct binding of Gli to DNA. However, GANT61 also inhibits the transcriptional factor ability of the Gli proteins and significantly decreases the expression of HH target proteins, inclusive of the HH signaling pathway

inhibitors (28). The suppression of Gli-1 has been observed to enhance the chemosensitivity of $CD34^{+}$-enriched AML progenitor cells, suggesting that Gli could be a potential target for AML treatments (9).

Numerous studies have investigated the inhibition of HH and mTOR signaling pathways due to their high potency as therapeutic targets for AML. Gli-1 and mTOR inhibition has been shown to induce apoptosis and demonstrated a synergistic effect in both solid tumors and hematological malignancies. In particular, GANT61 is seen to promote apoptosis in oral squamous cell carcinoma (OSCC) (28). Similarly, Miyazaki et al. (29) demonstrated the effect of the combination of GANT61 and rapamycin in pancreatic cancer stem cells, which showed an efficient reduction of 85% in cell proliferation. Moreover, this combination offered a decreased expression of the Gli-luciferase, along with the increased synergistic cytotoxic effect in different AML cells (30). A recent finding has also shown Gli-1, which is associated with poor prognosis in AML, to be found to activate the PI3K and cyclin-dependent kinases (CDKs) and thus promote the survival and cell-cycle progression. Synergistic inhibition of Gli-1 using GANT61 and CDK4/6 sensitized the cells to Ara-c therapy (31). Moreover, targeting of FLT3 wild-type AML cells with the combined FLT3, Gli-1, and PI3K inhibition resulted in a strong inhibitory effect on the FLT3 mutant AML cells (32). However, no reports are found concerning the effects of the combined GANT61 and rapamycin on both of those pathways in CMK or MOLM-13 cells. When compared with the literature, the results from the current study have proven a similar effect with a decrease in cell proliferation. The potential exists for a combination therapy approach to AML in the CMK and MOLM-13 cell lines by inhibiting the mTOR and HH signaling pathways using rapamycin and GANT61 in combination. The purpose of the present study has been to gain a better understanding of the effect of these two pathways in AML cells.

In agreement with the findings in the literature, the cell viability assays in the current study indicated the effect of the HH inhibitor GANT61 to decrease cell viability in a dose-dependent manner. However, the mTOR inhibitor rapamycin decreased cell viability by 50%, even though a high 1 µM dose of rapamycin had been tested on cells. The literature has shown that when a panel of AML cells is subjected to increasing concentrations of rapamycin and their clonogenicity is tested, some cell lines remain insensitive, which resulted in no change in colony numbers (26). Similar to this study's results, this effect could be due to the cytostatic effect of rapamycin. Combining rapamycin with other pathway inhibitors such as the PI3K/AKT pathway could sensitize the cells to rapamycin. Previous studies have also demonstrated reduced cell proliferation with regard to time and dosage (33, 34). For the combination treatment of the CMK cell line using the IC20 values for both Rapamycin and GANT61, a slight reduction occurred in cell proliferation when compared to the individual treatments. Therefore, the combined usage of these two drugs might prove to be more effective than when used individually. The differences in cell lines might also arise from the different genetic backgrounds of the cells. The cell cycle analysis revealed 17% of the CMK and 2.7% of the MOLM-13 cells to have been arrested during the G0/G1 phase in response to the combination treatment. The cell cycle arrest could be a reflection of the decreased cell proliferation in response to the drug treatment.

In summary, the present study proposes that targeting the mTOR and HH pathways simultaneously inhibits the cell proliferation of the CMK and MOLM-13 cell lines in AML. Moreover, the combinational treatment led to a G0/G1 cell cycle arrest, especially in the CMK cell line. Further in-depth studies should be conducted to understand the mechanistic effects of these drugs by evaluating the apoptotic effects for instance, with an elucidation of the underlying impact of these pathways on AML also being recommended.

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Ethics Committee Approval: In the current study, only *in vitro* cell line study was performed, therefore there is no need for ethical approval.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study - E.B.G.A.; Data Acquisition - E.B.G.A., F.M.K., E.C., M.Y.; Data Analysis/Interpretation - E.B.G.A., F.M.K., E.C., M.Y.; Drafting Manuscript - E.B.G.A., F.M.K., E.C., M.Y.; Critical Revision of Manuscript - E.B.G.A., F.M.K., E.C., M.Y.; Final Approval and Accountability - E.B.G.A., F.M.K., E.C., M.Y.

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