TÜBİTAK

# Akut Myeloid Lösemi Tedavisi için Hedgehog Ve Otofaji Yolaklarının Düzenlenmesi

**Program Kodu:** 3001

**Proje No:** 216S319

**Proje Yürütücüsü:**
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HAZİRAN 2019
KAYSERI

# PREFACE

The Project entitled “The fine tuning of the Hedgehog and autophagy pathways to combat Acute Myeloid Leukemia” is a 3001-project funded by TÜBİTAK. This project studies the effect of hedgehog signaling pathway and autophagy, a lysosomal degradation process, on the leukemogenesis of acute myeloid leukemia. The results obtained by this project will give us a better understanding that allows us to come up with more targeted therapy that selectively targets leukemic cells depending on their basal autophagic activity and the activation of the HH pathway.

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## ÖZET

Akut myeloid lösemi (AML) çeşitli moleküler aberasyonlar ve sinyal yolaklarındaki bozuklukları içeren klonal hastalıklar ile karakterize edilen bir grup heterojen malignanttır. Hedgehog (HH) sinyal yolağı birçok kanserde deregüle edilen evrimsel olarak korunan bir sinyal yolağıdır. HH sinyal yolağı lizozomal degradasyon prosesi otofajinin temel regülatörü olan PI3K/AKT/mTOR aksesini de içeren diğer sinyal yolakları ile karşılıklı iletişim halindedir. Bu sinyal yolakları AML'de deregüle edilmiştir. Birçok çalışmada otofajinin AML için bir kaçış mekanizması olabileceği ortaya konulmuştur. Bizim çalışmamızda, HH ve otofaji yolaklarının farklı AML türleri üzerine etkileri incelenmiştir. Çalışmamızda KML hücresi olan K562 ve CMK, MV4-11, MOLM-13 ve NB4 AML hücreleri GLI1 inhibitörü GANT61 ve farklı otofaji modülatörleri ile muamele edilmiştir.MTT sonuçları NB4, MOLM-13 ve MV4-11hücre proliferasyonun GLI inhibisyonu sonrasında düştüğünü ancak CMK'nin diğer AML hücre hatlarına kıyasla GLI inhibisyonuna daha az sensitif olduğunu ortaya koymuştur. Daha sonra, otofaji modülasyonunun farklı AML hücre hatlarının proliferasyonu üzerine etkileri incelenmiştir. Otofajinin gerek otofagozom-lizozom füzyonu aşamasında gerekse otofagolizozomal degradasyon aşamasında inhibisyonunun ilaç konsantrasyonu ve muamele süresine bağlı olarak AML sağkalımını azalttığı gözlemlenmiştir. Otofaji modülatörleri ve GANT61'in kombinasyonunun MOLM-13 hücre hattı üzerinde sinerjistik bir etkisinin olduğu fakat CMK hücre hattı üzerinde sinerjistik etkisinin olmadığı gözlemlenmiştir. GANT61 muamelesinin AML hücre hatlarında otofajiyi artırdığı LC3II ekspresyonu ile western blot yöntemi ile ortaya konulmuştur. Buna ek olarak, kombinasyonun MOLM-13 hücresinde LC3II'yi artırdığı gözlenirken, bu oran CMK hücre hattında daha düşüktür. AKT proteinin ekspresyonu ilaca ve hücre hattına gore farklılık göstermektedir. Sonuç olarak, HH ve otofaji sinyal yolaklarının hedeflenmesi MOLM-13 hücre hattı için umut vaatedici bir terapi iken, CMK hücre hattında benzer sonuçlara ulaşılamamıştır.

Anahtar Kelimeler: Akut myeloid lösemi, Hedgehog, Otofaji, PI3K/AKT/mTOR, kombinasyon terapi

## ABSTRACT

Acute myeloid leukemia (AML) is a heterogeneous group of malignancies characterized by clonal disorders with diverse molecular aberrations and dysregulation in signaling pathways. Hedgehog (HH) signaling pathway is an evolutionary conserved signaling pathway that is deregulated in many cancers. HH pathway crosstalks with other pathways among which is the PI3K/AKT/mTOR axes, a main regulator of autophagy, a lysosomal degradation process. These pathways are deregulated in AML. Several studies suggested that autophagy modulation could be an escape mechanism in AML. In this study, we investigated the effect of Hedgehog pathway and autophagy on different subsets of AML cell lines. We have treated CMK, MV4-11, MOLM-13 and NB4 AML cell lines, in addition to K562, CML cells, with GANT 61, GLI1 inhibitor, and different autophagy modulators. MTT assay have showed that GANT61 resulted in a significant decrease in the proliferation of NB4, MOLM-13, and MV4-11, however, CMK showed less sensitivity to GLI inhibition compared to other AML cell lines. Then, we proceeded with checking the effect of autophagy modulation on the survival of the different AML cell lines. Inhibiting autophagy whether at the autophagosome-lysosome fusion stage or autophagolysosomal degradation stage led to a decrease in the survival of AML to a certain degree based on the drug concentration and timepoint of treatment. Combination treatment of autophagy modulators and GANT61 had a synergistic effect in MOLM-13 but not in CMK. GANT61 treatment increased autophagy in AML cell lines that was correlated with an increase in the expression of LC3II detected by western blotting. Also, combination treatment elevated that increase in LC3II in MOLM-13 cell lines but less in CMK cell lines. AKT protein expression changed depending on the type of treatment and cell lines. In conclusion, targeting of HH and autophagy is a promising therapy against MOLM-13 cell line but not against CMK.



Keywords: Acute Myeloid Leukemia, Hedgehog, Autophagy, PI3K/AKT/mTOR, Combination therapy

# 1. INTRODUCTION

Acute myeloid leukemia (AML) is the most common adult acute leukemia with a low survival rate and a high resistance to chemotherapy. The main prognostic factor for AML was nonrandom cytogenetic molecular aberrations (Evangelisti et al., 2015; Ferreira et al., 2015). However, nowadays, there have been a reclassification of AML into new different risk groups based on the genetic mutation and molecular aberration. This was facilitated by the advance in genomic sequencing. The purpose of this study is to gain a better understanding of the molecular pathways that drive the pathogenesis of Acute Myeloid Leukemia (AML) in the hope of finding better treatment options for this deadly disease.

In this study we focus on the hedgehog signaling pathway and the pathways that regulate autophagy in the context of AML. The Hedgehog (HH) signaling pathway is an evolutionary conserved signaling pathway that is deregulated in many cancers. HH pathway crosstalks with other pathways among which is the PI3K/AKT/mTOR axes. This axis plays a pivotal role in the growth, metabolism, motility, proliferation, differentiation, survival, and angiogenesis of malignant cells (Jimenez-Sanchez et al., 2012; Milla et al., 2012; Pitralia et al., 2013; Sun et al., 2014). In general, it is known that HH inhibits autophagy (Jimenez-Sanchez et al., 2012; Milla et al., 2012; Pitralia et al., 2013). In addition to that, the PI3K/AKT/mTOR pathway regulates autophagy, a lysosomal degradation process that is essential for cellular homeostasis. Therefore, there is a kind of crosstalk and a regulatory loop that exists between these two pathways. Several studies indicated that these pathways are deregulated in acute myeloid leukemia. Moreover, Stankov et al. suggested that autophagy modulation could act as an escape mechanism in AML (Stankov et al. 2014).

Normally, AML maintains a basal level of autophagy that allows it to sustain cellular homeostasis and organelle turnover. We hypothesize that if we target AML with autophagy inhibitors in addition to inhibitors of the hedgehog signaling pathway, we could accomplish a synergistic effect that may provide a unique approach to target leukemic precursors in this disease based to their basal autophagy level and the expression of hedgehog genes. This is of particular importance since the hedgehog pathway have been shown to be dispensable for the maintenance of the normal adult hematopoiesis, while it is found to be deregulated in some AML patient sample. In accordance with that, targeting the HH pathway alone may not sensitize AML to hedgehog inhibitors and we hypothesize that this is due to the activation of autophagy upon HH inhibition. Thus, upon treating AML cells with inhibitors of HH in addition

to autophagy blockers we can accomplish a synergistic effect that can abolish AML. This understanding of the molecular pathways that drive the pathogenesis of AML will give us an insight into finding better treatment options for this deadly disease.

## 2. LITERATURE REVIEW

Acute myeloid leukemia is a clonal expansion of immature myeloid progenitors that are characterized by a block in differentiation and infiltration into the peripheral blood, bone marrow and other tissue such as the liver and the spleen (Döhner et al., 2015; Ferreira et al., 2015; Morita et al.,2015; Roloff & Griffiths, 2018; Bernand et al., 2019; Kayser & Levis, 2019). This results in the collapse of hematopoiesis, thus leading to anemia, neutropenia, and thrombocytopenia (Marelli et al., 2012).

AML constitutes around 80 percent of acute leukemias. It results in poor prognosis in adult patients with 80-90% of these patients relapsing (Marelli et al., 2012; Forristal et al., 2015; Roloff & Griffiths, 2018; Bernand et al., 2019; Kayser & Levis, 2019). On the other hand, younger patients present a better prognosis due to their high tolerance to chemotherapy (Marelli et al., 2012; Evangelisti et al., 2015). The combination of the chemotherapeutic drugs, cytarabine and anthracycline, led to a decrease in the number of leukemic blasts, however, this did not improve the prognosis of the patients (Bernand et al., 2019; Bhatt V. R., 2019). The main prognostic factor for AML was nonrandom cytogenetic molecular aberrations (Evangelisti et al., 2015; Ferreira et al., 2015). However, sequencing AML patients have expanded our knowledge about the pathogenesis of AML. This revealed the extensive heterogeneity of the disease and allowed the classification of AML patients into different risk groups depending on the molecular, genetic and epigenetic alterations (Marelli et al., 2012; Altman et al., 2014; Graubert et al., 2014; Döhner et al., 2015; Döhner et al., 2017; Evangelisti et al., 2015; Gu et al., 2018). These include t(8;21)(q22;q22) translocation, which is known as *AML1-ETO*, inv(16)(p13.1q22) inversion otherwise known as core binding factor *(CBF)B-MYH11* and 11q23 translocation that results in mixed lineage leukemia (MLL)- fusion proteins, in addition to that, t(6;9)(p23;q34)/*DEK-NUP214* and inv(3) (q21q26.2) or t(3;3) (q21;q26.2) translocations that result in *RPN1-EVI1* fusion (Evangelisti et al., 2015; Kayser & Levis, 2019). Other genetic mutations are being used as molecular markers in the prognosis of AML. One of them is the in frame internal tandem duplication of the juxtamembrane domain of the fms-like tyrosine kinase 3 (*FLT3*-ITD) (Gu et al., 2018; Garg et al., 2019; Daver et al., 2019). The other molecular diagnostic markers that have been recently added by World Health Organization (WHO) classification of AML are mutations in nucleophosmin 1 (*NPM1)* and CAAT enhancer-binding protein (*CEBPA*) (Graubert et al., 2014; Döhner et al., 2015, Evangelisti et al., 2015; Ferreira et al., 2015; Gu et al., 2018; Cocciardi et al., 2019; Bernand

et al., 2019; Garg et al., 2019; Kansal, 2019). Moreover, genetic mutations classify AML into different subgroups, among which is acute megakaryoblastic leukemia (AMKL) (Thiollier et al., 2012). AMKL is divided into down syndrome (DS)-AMKL and non-DS-AMKL depending on the genetic abnormalities. The feature of DS-AMKL is trisomy 21 associated with *GATA 1* (GATA binding protein 1 (globin transcription factor 1)) mutations, while non-DS-AMKL is characterized by the t(1;22)(p13;q13) chromosomal translocation (Thiollier et al., 2012; Kayser & Levis, 2019). Another subtype of AML is acute promyelocytic leukemia (APL) (Zeng et al., 2014). The clinical feature of APL is the Promyelocytic leukemia protein-Retinoic Acid Receptor, Alpha (*PML-RARA*) fusion gene resulting from t(15;17)(q24;q21) chromosomal translocation (Zeng et al., 2014; Kayser & Levis, 2019). Next generation sequencing of AML samples have allowed the identification of a number of new mutations in several geneses such as; DNA (cytosine-5)-methyltransferase 3A (*DNMT3A*), Tet Methylcytosine Dioxygenase 2 (*TET2*), Additional Sex Combs Like Transcriptional Regulator 1 (*ASXL1*), ETS-related gene (*ERG*), ecotropic viral intergration site 1 (*EVI1*), Meningioma 1 (*MN1*), Isocitrate Dehydrogenase 1 (*IDH1*), Isocitrate Dehydrogenase 2 (*IDH2*), Enhancer Of Zeste 2 Polycomb Repressive Complex (*EZH2*) and Brain And Acute Leukemia, Cytoplasmic (*BAALC.1*) (Döhner et al., 2015; Graubert et al., 2015; Morita et al., 2015; Gu et al., 2018; Young et al., 2019). In addition to that, genetic and molecular investigation of AML patients' samples that are collected throughout the treatment process from the initial presentation of the disease, the treatment period, remission and relapse have revealed the complex heterogeneity of AML (Graubert et al., 2015). It also revealed that subclones arise from a founder clone, which acquire new driving mutations with time (Graubert et al., 2015). This insight into the mutational background of AML recently resulted in the Food and Drug Administration (FDA) approving three targeted therapies against mutations in AML by the (FDA), which are midostaurin for *FLT3* mutations, enasidenib for relapsed or refractory cases with *IDH2* mutations, and ivosidenib for cases with an *IDH1* mutation (Bernand et al., 2019). This molecular profiling of AML gives some hope to this disease that is highly resistant to conventional chemotherapy (Wu et al., 2018).

In addition to the genetic abnormalities, aberrant signaling pathways could be the driver for the pathogenesis of AML. Some of the known aberrant pathways that are responsible for AML progression are the Hedgehog, Notch, PI3K/AKT/mTOR, WNT, Signal transducer and activator of transcription 3 (STAT3) and Tyrosine Kinase pathways (Heidel et al., 2015; Sakamoto et al., 2015; Liang et al., 2016; Ghosh & Kapur, 2017). These pathways crosstalk extensively, thus, promoting the pathogenesis of AML. There has been extensive evidence recently on the fact that AML modulate autophagy so that it acts as a survival and escape mechanism in the context of AML (Altman et al., 2014). Autophagy is a lysosomal degradation

process important in the maintenance of cellular homeostasis, organelle turnover, in addition to the regulation of metabolic processes (Jimenez-Sanchez et al., 2012; Martelli et al., 2012; Altman et al., 2014; Sun et al., 2014; Wang et al., 2014; Xu et al., 2014; Evangelisti et al., 2015).

Normally, cells maintain a low basal level of autophagy, which is activated physiologically by starvation or stress and chemically by rapamycin or trehalose (Jimenez-Sanchez et al., 2012; Milla et al., 2012; Evangelisti et al., 2015; Tomaipitinca et al., 2019). Depending on the cellular conditions and tissue context this degradative process has an important role in many diseases especially in cancer where it can impede or trigger and maintain tumor growth (Martelli et al., 2012; Milla et al., 2012; Altman et al., 2014; Wang et al., 2014; Xu et al., 2014; Evangelisti et al., 2015; Beljanski et al., 2019; Koustas et al. 2019; Tomaipitinca et al., 2019). The first step of autophagy is the induction and nucleation of the membrane that will become the autophagosomes, double- membrane vesicles. (Evangelisti et al., 2015; Beljanski et al., 2019; Tomaipitinca et al., 2019). These membranes are phagophores that include a part of the cytoplasm and engulf proteins, molecular aggregates, and impaired organelles. Autophagosomes then fuse to lysomomes, thus, leading to the formation of autophagolysosomes. These structures carry the enzymatic degradation of the engulfed material (Evangelisti et al., 2015 Beljanski et al., 2019; Koustas et al. 2019; Tomaipitinca et al., 2019). An important family of proteins that are essential and are involved sequentially in the autophagic process are the ‘Autophagy-related’ (ATG) proteins. These proteins form the main molecular machinery of autophagy (Evangelisti et al., 2015 Beljanski et al., 2019; Koustas et al. 2019; Tomaipitinca et al., 2019). Afterwards, elongation process starts allowing the autophagosome to fuse to the lysosome thus resulting in the formation of the autophagolysosomes, where its content is degraded by acid hydrolases and recycled by permeases (Evangelisti et al., 2015; Koustas et al. 2019; Tomaipitinca et al., 2019). The autophagic pathway is mainly regulated by mammalian target of rapamycin complex 1 (mTORC1) and AMP-activated protein kinase (AMPK) (Martelli et al., 2012; Evangelisti et al., 2015; Koustas et al. 2019). The mTORC1 is regulated by many upstream targets but the main regulator of this complex is the phosphoinositide 3-kinase (PI3K)/v-AKT murine thymoma viral oncogene (AKT) pathway (Evangelisti et al., 2015; Tomaipitinca et al., 2019). mTORC1 is a negative regulator of autophagy. It exerts this inhibitory effect by binding to UNC51-like kinase (ULK) complex which is composed of Ser/Thr protein kinase ULK1 (or ULK2), ATG13, focal adhesion kinase family-interacting protein of 200 kD (FIP200), and ATG101. The ULK complex is required for the initiation of the phagophore. In addition to that, the ULK complex can also be regulated directly by AKT (Evangelisti et al., 2015; Koustas et al. 2019; Tomaipitinca et al.,

2019). As mentioned before, AMPK is another main regulator of autophagy. Upon stress conditions, the tumor suppressor liver kinase B1 (LKB1) activates AMPK thus decreasing ATP consumption and induces ATP formation (Evangelisti et al., 2015; Koustas et al. 2019; Tomaipitinca et al., 2019). ULK1 is directly phosphorylated by AMPK at multiple sites, thus, leading to the activation of ULK1 (Evangelisti et al., 2015; Beljanski et al., 2019; Koustas et al. 2019). Therefore, AMPK unlike mTORC1 is a positive regulator of the autophagy (Evangelisti et al., 2015). Autophagy is a multistep process with a lot of proteins and lipids that come together to regulate this important physiological process. Beclin-1 core complex, which is composed of Beclin-1, the class III PI3K/hVps34, and p150/hVps15, is required for the autophagosome nucleation process (Evangelisti et al., 2015). Also, the elongation and degradation processes are regulated by two important ubiquitin-like conjugation systems. The initial system is made up of ATG7 and ATG3.These proteins controls the lipid modification of Light Chain 3 (LC3) (Evangelisti et al., 2015; Tomaipitinca et al., 2019). Lipidation of LC3 involves its cleavage by ATG4B. This converts cytosolic LC3-I to LC3-II, which is then bound to the autophagosome membrane, which is an important marker of an active autophagy (Evangelisti et al., 2015; Koustas et al. 2019). LC3 and p62 are important in sequestering proteins and organelles into the autophagosome (Evangelisti et al., 2015; Beljanski et al., 2019; Koustas et al. 2019). Thus, autophagy is a complex process regulated at so many levels by different proteins, which allows the chemical inhibition at the different levels of autophagy. This is achieved by pharmacological inhibition through the use of 3-Methyladenine (3-MA) which blocks the induction of LC3-II puncta formation, chloroquine (CQ) which is a lysosomotropic agent that has been suggested to inhibit autophagy by perturbing lysosomal function, also bafilomycin A1, or monensin can be used (Sun et al., 2014; Koustas et al. 2019). Another mode of inhibition is by the direct genetic silencing of ATG5, ATG7, Beclin-1, ATG10, or ATG12 thus, blocking autophagy (Sun et al., 2014).

In the recent years, several studies have shown the essential role of autophagy in AML. Stankov et al. have demonstrated that DS-AMKL cell lines and patient samples show a low basal level of autophagy due to the activation of the mTOR pathway by the aberrant insulin growth factor (IGF) signaling cascade (Stankov et al., 2014). They showed that by suppressing autophagy under a critical threshold, the leukemic growth is abrogated (Stankov et al., 2014). Moreover, recent studies have presented a lower expression of key autophagy- (ATG-) related genes in primary AML as compared to healthy granulocytes and human AML blasts showed reduced expression of autophagy genes Watson et al., 2015; Jin et al., 2018; Pereira et al., 2018). In addition to that, Sumitomo et al. have shown that autophagy is required for the maintenance of AML-initiating cells and peripheral myeloblast survival (Sumitomo et al., 2016).

Furthermore, a new axis, the (IRF2)–ino- sitol polyphosphate-4-phosphatase, type-II (INPP4B) axis, have been shown to induce autophagy in AML cell lines in order to prevent apoptosis and thus promoting the survival of AML (Zhang et al., 2019). To highlight the role of autophagy in the progression of myeloid leukemia, a study published by Nguyen et al. have shown that the loss of p62, an essential protein required in autophagy, led to the abrogation of murine myeloid leukemia progression and mitophagy (Nguyen et al., 2019). These studies suggest an important role for autophagy in the leukemogenesis of AML.

Many other subtypes of AML have shown an activation of the PI3K/mTOR/AKT pathway. In fact, this pathway has been shown to be activated in 95 percent of primary AML samples and 60-80 % in AML patients (Martelli et al., 2012; Bertacchini et al., 2015; Liang et al., 2016, Herschbein & Liesveld, 2017; Ricciardi et al., 2017; Li et al., 2019). The activation of the AKT pathway is an indicator of poor prognosis and decreased survival in AML patients due to the regulation of the expression of proteins associated with chemotherapy resistance, such as multidrug resistance-associated protein 1 (MRP1) and the membrane ATP-binding cassette (ABC) transporter (Wang & Zhong (2018)). Moreover, in hematopoietic stem cells, the activation of AKT is shown to be essential to their proliferation and if uncontrolled might lead to myeloproliferative disease, T-cell lymphoma, and leukemic transformation (Wang & Zhong (2018)). Thus, targeting this pathway is an effective therapeutic option (Ricciardi et al., 2017). Furthermore, signals from the AML microenvironment such as the C-X-C motif chemokine ligand (CXCL)8 (CXCL8) have shown to promote the growth AML cells through the activation of the PI3K/AKT pathway (Cheng et al. 2019). However, some studies have shown that the activation of the PI3K/AKT pathway in *de novo* AML patients have led to better prognosis (Wang & Zhong (2018)). In addition to that, the downfall of the inhibition of the PI3K/mTOR/AKT pathway could lead to the activation of the autophagic machinery as a result of mTOR inhibition (Martelli et al., 2012; Aronson et al. 2013; Altman et al., 2014). Thus, indirectly promoting the pro-leukemic effect of autophagy in the AML patients (Martelli et al., 2012; Aronson et al. 2013; Altman et al., 2014). This suggests autophagy as an escape mechanism of chemotherapeutics or anti-leukemic agents and targeting autophagy especially in p53 wild-type acute myeloid leukemia could be a promising treatment strategy for these patients (Folkerts et al., 2017). Several studies have shown that this can be overcomed by combinational therapies comprising of both mTOR inhibitors and autophagy blockers (Martelli et al., 2012; Aronson et al. 2013; Altman et al., 2014). The anti-leukemic effects of autophagy inhibition were also shown in AML-ETO samples (Evangelisti et al., 2015, Kühn et al., 2015; Man et al., 2017). In addition to that, *FLT3*-ITD mutations have been found to increase the

basal autophagy in AML cells through transcription factor ATF4, thus, promoting the initiation and progression of AML with *FLT3*-ITD mutations (Heydt et al., 2018). Moreover, Atg5-dependent autophagy was linked to the development of acute myeloid leukemia in an MLL-AF9-driven mouse model (Liu et al., 2017). However, in the context of APL, autophagy has been reported to be crucial for the anti-leukemic effects of arsenic trioxide and all-trans-retinoic acid (ATRA) treatment (Zen et al., 2014, Altman et al., 2014; Schläfli et al., 2017). This is due to the fact that PML-RARA protein aggregates are degraded by the autophagy-lysosome pathway (Zen et al., 2014, Altman et al., 2014). However, a recent study by Lui et al. has shown that combined treatment of chloroquine with arsenic trioxide (As2O3) induced the apoptosis of acute promyelocytic leukemia, NB4 cells, further via inhibiting lysosomal degradation in vitro (Lui et al., 2018). It has also been shown that autophagy plays a role in the transition of myelodysplastic syndrome to AML (Evangelisti et al., 2015). Moreover, recent studies have shown that autophagy inhibition sensitized AML cells to Ara-c and Sorafenib treatment (Kim et al., 2015; Guo et al., 2018). Furthermore, Hu et al., have shown that autophagy can be regulated by the stromal cell-derived factor 1α-CXC chemokine receptor type 4 (SDF-1α-CXCR4) signaling axis, where CXCR4 surface expression is considered an independent prognostic factor for disease relapse and survival in acute myeloid leukemia (AML) patients (Hu et al., 2018). ATG7 expression in AML patients have been linked to shorter remission and a study have shown that ATG7 knockdown led to increased apoptosis and DNA damage following treatment with cytarabine and idarubicin. This suggests that ATG7 specifically and autophagy generally plays an important role in AML chemoresistance (Piya et al., 2016). A recent study by Nguyen et al. has shown that the loss of the autophagy receptor, p62, led to impaired murine myeloid leukemia progression and mitophagy (Nguyen et al., 2019). On the other hand, in mt2a/Mll- Mllt3/Af9 AML (MA9-AML) cells, targeting autophagy had no effect on the growth or survival of this subtype of AML (Chen et al., 2017). The role of autophagy in AML leukemogenesis is controversial, another publication has shown an important role of autophagy in AML growth inhibition, where they have shown that RET-mTORC1 signaling promotes AML through autophagy suppression (Rudat et al., 2018). Thus, a thorough understanding is required to understand the role of autophagy in AML as an anti-leukemic or a pro-leukemic mechanism, which depends on the cellular context and the molecular aberrations leading to this leukemia. Clinical trials for autophagy blockers in combination with known chemotherapeutic drugs have already been initiated and showing promising results (Evangelisti et al., 2015). Therefore, gaining a better insight into the autophagic pathway and how it crosstalks with other pathways allows to develop a more personalized therapy, which results in the greatest benefit for the different AML patients (Liang et al., 2018).

Many signaling pathways crosstalk and regulate autophagy. One of these signaling pathways is the HH signaling pathway. It is an evolutionary conserved signaling pathway playing a role in many cell-determining processes especially during development and embryogenesis (Cilloni and Saglio, 2012; Ghezali et al., 2014; Wellbrock et al., 2015; Zuo et al., 2015; Didiasova et al., 2018; Ghirga et al., 2018; Sari et al., 2018; Bariwal et al., 2019, Niewiadomski, 2019; Salaritabar et al., 2019). Hedgehog is also involved in tissue polarity, differentiation and stem cell maintenance (Cilloni and Saglio, 2012; Ghezali et al., 2014; Wellbrock et al., 2015; Zuo et al., 2015). Aberrations in this pathway have been shown to be a main oncogenic driver in many cancers such as glioblastoma, medullobalstoma, cholangiocarcinoma, pancreatic cancer, basal cell carcinoma, breast cancer, cervical cancer and some hematologic malignancies and in several other cancers (El Khatib et al., 2013; Zuo et al., 2015; Sari et al., 2018; Ghirga et al., 2018; Bariwal et al., 2019; Hanna et al., 2019; Salaritabar et al., 2019; Sharma et al., 2019). The canonical Hedgehog pathway is activated upon binding of the HH ligands: Sonic hedgehog (SHH), Indian Hedgehog (IHH) and Desert Hedgehog (DHH) to the transmembrane receptor patched (PTCH) (Ghezali et al., 2014; Heidel et al., 2015; Kern et al., 2015; Sadarangani et al., 2015; Wellbrock et al., 2015; Ghirga et al., 2018; Sari et al., 2018; Niewiadomski, 2019). Thus, relieving the inhibitory effect of PTCH on smoothened (SMO), the HH effector. This results in the translocation of SMO to the primary cilium, thus its activation and the initiation of the downstream signaling cascade (Ghezali et al., 2014; Heidel et al., 2015; Kern et al., 2015; Sadarangani et al., 2015; Wellbrock et al., 2015; Ghirga et al., 2018; Sari et al., 2018; Niewiadomski, 2019). This allows the Glioblastoma (GLI) family of transcription factors to translocate to the nucleus and activate their target genes involved in proliferation, migration, invasion, metastasis and angiogenesis (Ghezali et al., 2014; Heidel et al., 2015; Kern et al., 2015; Sadarangani et al., 2015; Wellbrock et al., 2015; Niewiadomski, 2019). The hedgehog pathway can also be activated non-canonically. In some human malignancies, GLI has been shown to be regulated independently of SMO. Atypical protein kinase C (aPKC), transforming growth factor beta (TGF-β)/SMAD, and the Ewing's sarcoma *EWS/FLI-1* oncogene have been described to regulate GLI (Kern et al., 2015; Zuo et al., 2015). Also, tyrosine kinases have been shown to activate HH such as the RAS/RAF/MEK/ERK and PI3K/AKT/mTOR pathways (Kern et al., 2015; Zuo et al., 2015 Sari et al., 2018). Multiple non-canonical pathways involved in tumor growth can increase GLI activity. Moreover, it can be activated noncanonically by K-RAS, TGF-β and PI3K-AKT, however the mechanisms of activation are still ambiguous (Ghirga et al., 2018, Zeng & Ju, 2018).

The crosstalk between hedgehog and the PI3K/AKT/mTOR pathway has been shown in many cancer studies, thus presenting the outcome of the interaction of these pathways on autophagy

(Sun et al., 2014). In general, it is known that HH inhibits autophagy (Jimenez-Sanchez et al., 2012; Milla et al., 2012; Pitralia et al., 2013). Jimenez- Sanchez et al. have shown that HH blocks autophagy by negatively regulating the phosphorylated extracellular signal-regulated kinases – eukaryotic transcription factor alpha (pERK- eIF2α) (Jimenez-Sanchez et al., 2012; Pitralia et al., 2013).

GLI down-regulation has been shown to induce autophagy by regulating the mTOR phosphorylation through the ERK1/2 (Sun et al., 2014). In neuroblastoma and pancreatic cancer, HH inhibition leads to the induction of autophagy that has been shown to be the reason the resistance to HH inhibitors in these tumors (Milla et al., 2012; Wang et al., 2014; Xu et al., 2014). In the context of breast cancer, a study showed that HH inhibition led to the induction of apoptosis and autophagy, however autophagy was a pro-death mechanism in breast cancer (Wang et al., 2017). This phenomenon was also detected in hepatic stellate cells, Hedgehog signaling inhibitor GANT61 led to the induction of endoplasmic reticulum stress-mediated protective autophagy, in addition to that, miR-148a induced autophagy through the SHH pathway (Liu et al 2015; Li et al., 2017). The autophagic pathway is directly regulated by HH. A study that was done by Milla et al. showed the existence of multiple GLI consensus binding sites on the human ATG5 promoter (Milla et al., 2012). The combination treatment of HH and autophagy inhibitors have been shown to be a promising therapeutic option against B-cell non-Hodgkin's lymphoma (Fan et al., 2016). In addition to that, recently, a study by Okuhashi et al. have shown that HH and WNT pathways are upstream of mTOR and NOTCH pathways and downregulate them in NB4 acute promyelocytic leukemia cell line (Okuhashi et al., 2018). On the other hand, some studies indicated that HH signaling leads to the upregulation of autophagy (Milla et al. 2012, Zeng et al. 2018). Moreover, in some cancers, oncogenic KRAS have been shown to activate autophagy to promote tumorigenesis and cancer progression through the KRAS–PI3K–AKT1–GLI3–VMP1 axis (Zeng et al., 2018). Interestingly, a recent study by Chen et al. have detected a noncanonical function of PATCHED-1, where it inhibits autophagy by interacting with ATG101. (Chen et al., 2018)

Moreover, the opposite is also true; an activated mTOR pathway leads to GLI transcriptional activation (Sun et al., 2014). PI3K/AKT/mTOR axis also regulates GLI activity through protein phosphatase 2A (PP2A), a phosphatase that acts as an antagonist to the mTOR complex. PP2A have been shown to prevent the nuclear localization of Gli3 (Brechbiel et al., 2014; Wellbrock et al., 2015). Several studies have documented the crosstalk between the PI3K/AKT and the MEK/ERK pathways to promote epithelial to mesenchymal transition (EMT), metastasis, and angiogenesis through GLI activation (Brechbiel et al., 2014; Kern et al., 2015).

Liang et al. have found that the upregulation of GLI1 led to increased AKT phosphorylation and decreased drug sensitivity in AML cells which was attenuated by GLI1 inhibition (Liang et al., 2017)

In addition to that, to the known oncogenic role of HH in solid tumors, it has been implicated in the pathogenesis of many leukemias. However, its importance in hematopoiesis is controversial (Hofmann et al., 2009; Heidel et al., 2015). Several studies have shown that the HH pathway is dispensable to the normal adult hematopoietic development (Hofmann et al., 2009; Heidel et al., 2015; Pession et al., 2019). However, these studies don't eliminate the role of HH in many hematological malignancies. For instance, the aberrant activation of the Hedgehog pathway has been shown to be essential for the maintenance of some CD34+ leukemia stem cells (Shafer & Grant, 2016; Pession et al., 2019). The aberrant HH signaling pathway maintains the leukemic stem cell (LSCs) populations by the direct binding of GLI to *NANOG* promoter, thus promoting the self-renewal and stemness properties of these cells (Kakiuchi et al., 2017). Lau et al. recently showed that Hedgehog activation by GLI1 led to the leukemic transformation of myelodysplastic syndrome in vivo the inhibition of this pathway by GANT61 resulted in antileukemic activity (Lau et al., 2019). Moreover, leukemias with activated breakpoint cluster region (BCR)- Abelson murine leukemia viral oncogene homolog 1 (ABL) kinase have shown an activation of the HH pathway (Hofmann et al., 2009; Cilloni and Saglio,2012; Brechbiel et al., 2014). Zhao et al. have also shown the importance of HH in the self-renewal capacity of stem cells in chronic myeloid leukemia (CML) (Hofmann et al., 2009; Brechbiel et al., 2014; Heidel et al., 2015; Sadarangani et al., 2015,). In addition to that, a study done by Zeng et al showed that the targeting HH signaling pathway and autophagy overcomes drug resistance of BCR-ABL-positive CML (Zeng et al., 2015). Also, HH have been shown to promote oncogenic signals in chronic lymphocytic leukemia (CLL) (Kern et al., 2015; Sadarangani et al., 2015; Zeng et al., 2015).

In the context of AML, several studies have shown that chemotherapeutic resistance to be attributed to aberrant HH signaling (Sadarangani et al., 2015). A study done by Zahreddine et al. showed that GLI1 and UDP glucoronosyl-transferase (UGT1A) family of enzymes are over expressed in Ara-C resistant leukemia and that upon the inhibition of GLI1, leukemic growth was abrogated (Zahreddine et al., 2014; Sadarangani et al., 2015). AML patients express PTCH, SMO and the downstream targets of the HH pathway; GLI1, GLI2, and GLI3 (Wellbrock et al., 2015). GLI2 expression was found to be negatively correlated with the survival rate of AML patients (Wellbrock et al., 2015, Bouscary, 2016). It was also highly detected in relapsed patients (Wellbrock et al., 2015). In addition to that, it was connected to the *FLT3*- mutational status (Wellbrock et al., 2015; Pession et al., 2019). An increase in GLI2 expression was

detected in *FLT3*-ITD AML (Shafer & Grant, 2016; Aberger et al., 2017). Thus, GLI2 have been suggested as a negative prognostic marker in AML patients (Wellbrock et al., 2015). In addition to that, a recent study done by Latuske et al. showed that the combination treatment GLI and FLT3 signaling leads to promising anti-leukemic effects in AML (Latuske et al., 2017). Hedgehog pathway plays a role in megakaryocytic differentiation whereby blocking HH led to the inhibition of ERK1/2 pathway, which is an important pathway, involved in the megakaryocytic differentiation (Ghezali et al., 2014). This was also confirmed in CD34 positive (CD34+) cells, which expressed HH when they were undergoing hematopoietic differentiation. In addition to that, deregulated HH pathway was detected in in MDS and AML patients (Khan et al., 2015; Pession et al., 2019). AML is a cytogenetically aberrant disease and some of these fusion genes belong to the hedgehog pathway genes. One example is the *CBFA2T3-GLIS2* fusion gene, which is formed by the fusion of *CBFA2T3*, a member of the ETO family of nuclear corepressors, and *GLIS2*, a member of the GLI family of transcription factors, and it is detected in non-Down syndrome acute megakaryocytic leukemia (AMKL), as well as in some other non-megakaryoblastic AML subtypes (FAB-M0, M1, M2, M4, M5, M5a) (Masetti et al, 2017; Mitsui-Sekinaka, 2018). Patients with *CBFA2T3-GLIS2*-positive AMKL have very poor survival rate (Masetti et al, 2017; Mitsui-Sekinaka, 2018). Another example is the Desert Hedgehog-RAS Homologue Enriched in Brain Like 1 (*DHH-RHEBL1*) is fusion gene, which was detected in *BFA2T3-GLIS2*-positive leukemic cells (Mitsui-Sekinaka, 2018). Moreover, ATO, which is an effective treatment against APL, has been shown to effectively suppress the SHH pathway by downregulating the expression of GLI (Carpenter &· Ray, 2019). The expression of GLI in the context of AML have been shown to be able to induce the UGT1A-dependent glucuronidation of ribavirin and cytarabine, thus leading to resistance to chemotherapy in this leukemia (Sabol et al. 2018). The chemoresistance due to the upregulation in the hedgehog pathway is believed to be imparted by the B4GALT family members (Savona et al., 2018). Therefore, targeting the HH signaling pathway has been viewed as prospective antileukemic therapy (Savona et al., 2018). In addition to that, blocking the hedgehog pathway in AML cell lines resulted in a decrease P-glycoprotein expression and multidrug resistance. Moreover, multiple studies have indicated that hedgehog inhibition have sensitized AML to multiple chemotherapeutic drugs such as; cytarabine, 5-azacitidine, or radiotherapy (Savona et al., 2018). In addition to that, in refractory AML, GLI-1/PI3K/AKT/NF-kB pathway was shown to be a major player in in resistance to radiation and blocking the HH pathway led to the sensitization of these AML cells to radiation thus conquering radioresistance (Li et al., 2016; Aberger et al., 2017). Recently, a novel HH pathway inhibitor, Glasdegib (PF-04449913), is in clinical development for patients with myeloid malignancies where its shown to decrease the LSC populations in cells from AML patients and it presents itself as a promising option for AML patients, which are not suitable for

intensive chemotherapy (Savona et al., 2018; Cortes et al., 2019). Fukushima et al., showed that targeting the hedgehog pathway led to blocking the leukemia initiation potential of AML (Fukushima et al., 2016). As a result of all of these studies, in November 2018, the Food and Drug Administration (FDA), approved the use of glasdegib in combination with low-dosage of cytarabine as a treatment regimen for the newly-diagnosed acute myeloid leukemia (AML) patients who are above 75 years (Hoy S., M., 2019; Norsworthy et al. 2019). In conclusion, targeting the hedgehog pathway in AML might be promising especially due to the fact that HH has a dispensable role in adult hematopoiesis, which offers a therapeutic window by targeting specifically leukemic cells and not the normal hematopoietic cells. However, HH inhibition alone is unlikely to be successful especially due to the complexity of this pathway and its crosstalk with other pathways. In addition to that, HH inhibition as mentioned before leads to the activation of autophagy, which is a survival and escape mechanism in AML. Thus, understanding the interaction of HH and autophagic pathways in the context of AML might provide an insight into the mechanism that leads to the pathogenesis of AML. This would lead to a more specific combinational-targeted therapy resulting in the abrogation of AML.

## 3. MATERIALS AND METHODS

Cell Maintenance: The experiments were performed on AML cell lines originating from different AML subgroups:

- MOLM-13 and MV4-11 are AML M5 cell line with an *FLT3*-ITD mutation.
- NB4 is a *PML-RARA* AML cell line.
- CMK are AML M7 cell line from down-syndrome patients.

In addition to that, K562, a chronic myeloid leukemia (CML) cell line, was used as a control cell line since it has been shown that the inhibition of hedgehog and autophagy is cytotoxic in K562 and CML (Zeng et al., 2015).

All cell lines were cultured in RPMI medium supplemented with 10 % FBS except CMK was cultured in 20 % FBS and 100 U/mL penicilin/streptomycin at 37 C in 5% $CO_2$. These cell lines were purchased from the German National Resource Center for Biological Material (DSMZ), and were cultured under the recommended conditions (Klusmann et al., 2010; Stankov et al., 2014).

Detection of Protein Expression by Western Blotting: Cells were collected and rinsed with cold phosphate-buffered saline (PBS). Then harvested cells were lysed in RIPA lysis buffer system (ChemCruz, cat.no. sc-24948) was combined with 10µ protease inhibitor cocktail,10µ PMSF solution and 10µ sodium orthovanadate solution per ml of 1X RIPA lysis buffer. The concentration of extracted protein was determined using DC protein assay kit (Biorad/USA cat. no. 500-0113, cat.no. 500-0114, cat. no. 500-0115) following manufacturer's instruction. The absorption was measured at 750 nm using a microplate reader. For immune blotting the cell lysates were loaded at a protein concentration of 60µg per well. Gel electrophoreses (10-15% acrylamide gels) were performed (Biorad, München). The membranes were blocked using 5% dried milk in 1xTNT (5M NaCl, 2M Tris pH 7,5 and 10 % Tween20) 1 hr at room temperature and incubated overnight at 4°C with the primary antibodies. The following antibodies were used: ULK1 (1:1,000; cat. no. 4773S; Cell signaling), AKT (1: 1,000; cat. no. 4691S; Cell signaling), LC3B (1:1,000; cat. no. 2775S; Cell signaling) and GAPDH (1:1,000; cat. no. 2118S; Cell signaling) and Anti-β-ACTIN(1:1,000; cat. no. A1978; sigma aldrich) were used as a loading control. Membranes were incubated with the following secondary antibodies for 1hr at room temperature: Peroxidase AffiniPure Goat Anti-Rabbit IgG (H+L) (1:10,000 - 1:200,000 for Western blotting with ECL substrates; cat. no. 111-035-003); Peroxidase AffiniPure Goat Anti-Mouse IgG (H+L) (1:10,000 - 1:200,000 for Western blotting with ECL substrates; cat. no. 115-035-003) (both from jackson immunoresearch europe ltd). The signals were detected by Pierce™ ECL Western Blotting Substrate (cat. no: 32106; UK) with ChemiDoc™ Imaging Systems (Biorad).

Drug Preparation and In Vitro Cell Viability Assay: In order to check whether the hedgehog and autophagy pathways are activated in AML, the different AML cell lines were divided into different treatment groups:

1- Dimethysuldoxide (DMSO) or water
2- Hedgehog pathway inhibitors (GANT61)
3- Autophagy pathway blockers
4- Combination of Hedgehog and autophagy pathway inhibitors

Stock solutions of Gli inhibitor, GANT61 [[dihydro-2-(4-pyridinyl)-1,3(2H,4H)-pyrimidinediyl]bis (methylene)]bis[N,Ndimethyl benzenamine] (Stanton et al., 2009; Desch et al., 2010; Ozone et al., 2010; Queiroz et al., 2010; Amakye et al., 2013; Kakanj et al., 2013; Zahreddine et al., 2014; Han et al 2015; Infante et al., 2015; Kern et al 2015; Samanta et al., 2015; Falkenberg et al 2016; Vlčková et al., 2016). Cells were treated with DMSO and GANT61 (1µM-20µM) and

were analyzed after 24, 48, and 72 hours to determine the IC20, IC30 and IC50 concentrations. IC50, IC30 and IC20 values were determined by Graphpad prism 7 program based on the proliferation curve (El Khatib et al., 2013; Ghezal et al., 2014). Also, the autophagy pathway was manipulated at different levels by different autophagy blockers. Autophagy manipulation was performed in accordance to the recently updated guidelines that were published in Cell (Mizushima et al., 2010). Activation was achieved by using PP242 (0.01- 0.05μM). Inhibition of autophagy was achieved as follows: autophagosome-lysosome fusion using vinblastine and nocodazole (0.01 to 50μM); autophagolysosomal degradation using $NH_4Cl$ (0.5 to 20mM), chloroquine and hydroxychloroquine (5 to 100μM).

Cell proliferation was determined using MTT Cell Viability Assay. For this purpose, cells were seeded in triplicate in 96-well plates at a $10^4$ cells per each well. At the indicated time-points (24, 48, 72 h), 10 *μ*l of MTT solution (cat. no. M2128; Sigma aldrich) was added to each well and the plates were incubated for 2 h at 37°C. After 2h incubation, 100 *μ*l of DMSO were added to each well to solubilize the crystals. The plates were shaken for 15 min and the absorbance was measured with a Varioskan™ LUX multimode microplate reader (Thermo Scientific™) at 570 nm.

RNA extraction, semi-quantitative PCR and Real Time-Quantitative PCR These techniques were used to check the effectiveness of the inhibition of the hedgehog pathway by Cyclopamine and GANT61:

RNA was extracted using PureLink™ RNA Mini Kit (cat. no. 12183018A, Invitrogen). The cDNA synthesis was performed using High-Capacity cDNA Reverse Transcription Kit (cat. no. 4368814, Applied Biosystems) from 2μg total RNA concentration. Semi- quantitative PCR were performed for *GLI1* (F5' CTCCCGAAGGACAGGTATGTAAC'3/R5'CCCTACTCTTTAGGCACTAGAGTTG'3) (Kobune et al., 2009; Sing et al., 2010) *GLI2* (F5'TGGCCGCTTCAGATGACAGATGTTG'3/R5'CGTTAGCCGAATGTCAGCCGTGAAG'3) (El Khatib et al., 2013), *SMO* (F5'GTTCTCCATCAAGAGCAACCAC'3/R5'CGATTCTTGATCTCACAGTCAGG'3) (El Khatib et al., 2013), and 18-sRNA (F5'AAACGGCTACCACATCCAAG'3/R5' CCTCCAATGGATCCTCGTTA'3) (El Khatib et al., 2013) with product sizes of 248, 200, 258, and 155 respectively. PCR amplification conditions for GLI1 and 18-sRNA were: initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 56°C for 30 sec, annealing at 55°C for 45 sec, and elongation at 72°C for 2.30 sec; and final elongation at 72°C for 5 min. PCR amplification conditions for GLI2 were: initial denaturation at 95°C for 5 min; 35 cycles of

denaturation at 65°C for 30 sec, annealing at 65°C for 45 sec, and elongation at 72°C for 2.30 sec; and final elongation at 72°C for 5 min. PCR amplification conditions for SMO were: initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 65°C for 30 sec, annealing at 59°C for 45 sec, and elongation at 72°C for 2.30 sec; and final elongation at 72°C for 5 min. The primers used are already published primers and for more confirmation regarding their specificity, they were blasted and found to be highly specific for their respective genes. After amplification, the PCR products were loaded to 2% (W/V) agarose gel (1× TBE buffer) containing 4 µl ethidium bromide for electrophoresis. After running, gels were imagined with Gel Doc™ XR+ Gel Documentation System (Biorad).

Autophagy Modulation by Autophagy Blockers: The autophagy pathway was manipulated at different levels by different autophagy blockers. Autophagy manipulation was performed in accordance to the recently updated guidelines, which were published in Cell (Mizushima et al., 2010). Activation was achieved by using Activation was achieved by using PP242 (0.01-0.05µM). Inhibition of autophagy was achieved as follows: autophagosome-lysosome fusion using vinblastine and nocodazole (0.01 to 50µM); autophagolysosomal degradation using $NH_4Cl$ (0.5 to 20mM), chloroquine and hydroxychloroquine (5 to 100µM). Autophagic activity was measured in accordance to the recently updated guidelines (Mizushima et al., 2010). We checked the protein expression of the autophagy markers: ULK-1 and LC3B. The following antibodies were used for Western blot analysis: LC3B, ULK-1, AKT, ATG5, GAPDH and Anti-β-ACTIN and the above mentioned experimental procedure for western blot was followed to detect the expression of these autophagic markers.

Cell Death Assay: For apoptosis assay, cells were harvested in 6 well plate for 48h incubation time. After incubation, cells were collected and centrifugate at 1700 rpm for 5 minutes. Then, cells washed with phosphate-buffered saline (PBS) twice. After centrifugation, cells were dissolved in 200 µl binding buffer. Apoptosis was measured after double staining with Annexin V FITC/PI (eBioscience™ Annexin V Apoptosis Detection Kit APC, cat. no. 88-8007-72) with Flow cytometer.

Statistical analysis: The statistical analyses were done by unpaired Student's t-test. Comparisons of more than two groups were performed by 2-way ANOVA with Dunnett's multiple comparisons test and the significance of the test was evaluated as $p < 0.05$ by using

GraphPad Prism 8 (ns=P > 0.05, * =P ≤ 0.05, **= P ≤ 0.01=, *** P ≤ 0.001, **** =P ≤ 0.0001). All data were presented as mean ±s.d.

## 4. RESULTS AND DISCUSSION

### 4.1 Inhibition of GLI 1, a player in the HH pathway, by GANT 61 leads to a decrease in the proliferation of AML cell lines:

In order to access the importance of HH pathway on AML cell lines, we performed MTT assay after treating the different AML cell lines with different concentrations of GANT61 for 24, 48 and 72 hrs. We plated 10,000 cells per well for each cell line (CMK, MOLM-13, NB4, MV4.11 and K562) and treated these cells with vehicle control (DMSO) and increasing concentration of GANT61 ranging between 0.5 to 20 µM for MV4.11 and NB4 and 5 to 20 µM for CMK, MOLM-13 and K562 cell lines at different time points (24, 48 and 72 hrs). There was a significant decrease in the proliferation of MV4.11 at 2.5, 5, 10 and 20 µM at the different time points (24, 48 and 72hrs) (Figure 1).

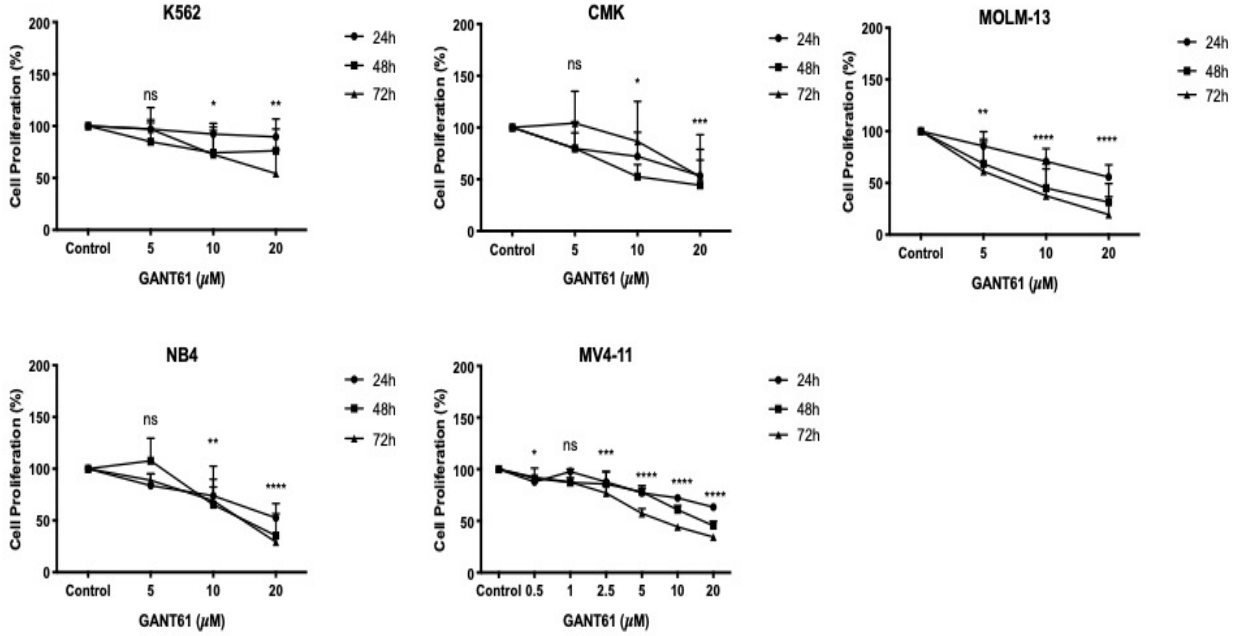


Figure 1. Blocking of the Hedgehog signaling pathway using GANT61 decreases cell proliferation of AML cell line (CMK, MOLM-13, NB4 and MV4-11) at different time points (24, 48 and 72hrs).
K562, CML cell line was used as a control. The number of replicates was 3 in each experiment and the average of the replicates performed during each experiment were imported to GraphPad, which is 3 wells per treatment. The independent experiments were done three times (n=3) for K562, four times (n=4) for CMK, five times (n=5) for MOLM-13, three times (n=3) for NB4 and twice (n=2) for MV4-11. The statistical analyses and the comparisons of more than two groups were performed by 2-way ANOVA with Dunnett's multiple comparisons

test and the significance of the test was evaluated as p <0.05 by using GraphPad Prism 8. (ns=P > 0.05, * =P ≤ 0.05 , **= P ≤ 0.01=, *** =P ≤ 0.001, **** =P ≤ 0.0001). All data are presented as mean±s.d.

NB4 cell line also showed a significant decrease at 10 and 20uM of GANT treatment at 24, 48 and 72 hrs (Figure 1). In addition to that, GANT61 treatment of CMK cell line led to a significant decrease in its proliferation after 24hrs and 48hrs for 10 and 20μM. CMK cells showed more proliferation at 72hrs of GANT treatment compared to 24 and 48 hours especially at lower doses (10 μM) but this increase in proliferation was abrogated at 20 μM GANT (Figure 1). As for MOLM-13 cell line, GANT61 significantly decreased the proliferation of these cells at 5, 10 and 20μM at 24,48 and 72 hrs (Figure 1).

As expected, K562 cell line was more resistant to GANT61 treatment compared to AML cell lines. The significant decrease in proliferation was only observed 10 and 20 μM GANT61 treatment for 48 and 72 hrs (Figure 1).

These results indicate that AML cell lines are sensitive to GANT 61 treatment and that blocking the hedgehog pathway can be a potential therapeutic option to AML. Our data confirm previous studies done K562 and on other AML cell lines such as Kasumi-1, HL-60, and U937 (Pan et al., 2012). Another study has shown that the CD34+ cells were more sensitive to GANT61 inhibition in comparison to cells that have a lower CD34 expression. It would be interesting to check the CD34+ status of our AML cell lines to check if their response to GANT61 inhibition would vary with their CD34 expression (Long et al. 2016). Our results have shown that CMK, acute megakaryoblastic leukemia cell line, was more resistant to GANT 61 treatment, GLI1 inhibitor, one explanation could be that GLI1 is not the most important member of the hedgehog family that leads to the leukemogenesis of CMK and other members such as GLI2 could play a bigger role in the pathogenesis of CMK. Interestingly 17 % of non-Down syndrome acute megakaryoblastic leukemia have a *CBFA2T3-GLIS2* fusion gene, which leads to the activation to the hedgehog pathway, thus it would be interesting if there is an over-expression of GLI2 in CMK and whether CMK possess this fusion gene (Masetti et al., 2017).

**4.2 Autophagy inhibition decreases the proliferation of AML cell lines:**

To understand the importance of autophagy in AML we aimed to manipulate the pathway at different levels by different autophagy blockers. Autophagy manipulation is performed in accordance to the updated guidelines by Mizushima et al., which were published in Cell (Mizushima et al., 2010). Inhibition of autophagy is achieved as follows: autophagosome-lysosome fusion using vinblastine and Nocodazole; autophagolysosomal degradation using

ammonium chloride ($NH_4Cl$), chloroquine (CQ) and hydroxychloroquine (HQ). Autophagy manipulation allows us to see the importance of autophagy to AML cell lines and whether it is essential to sensitize AML cell lines to HH inhibition.

In order to check the effect of the autophagy inhibitors we performed MTT assay after treating the different AML cell lines with different concentrations of the autophagy inhibitors for 24, 48 and 72 hrs. We plated 10,000 cells per well for each cell line (CMK, MOLM-13, NB4, MV4.11 and K562) and treated these cells with vehicle control (Water) and increasing concentration of the inhibitors (vinblastine; $NH_4Cl$, chloroquine (CQ) and hydroxychloroquine (HQ)).

We wanted to check the effect of the inhibition of blocking the autophagosome-lysosome fusion on AML cell for that we used vinblastine and nocodazole. Vinblastine treatment (10-50 μM) led to a significant decrease in the proliferation of CML cell line, K562, and AML cell lines CMK, MOLM-13, MV4.11 and NB4 (Figure 2).

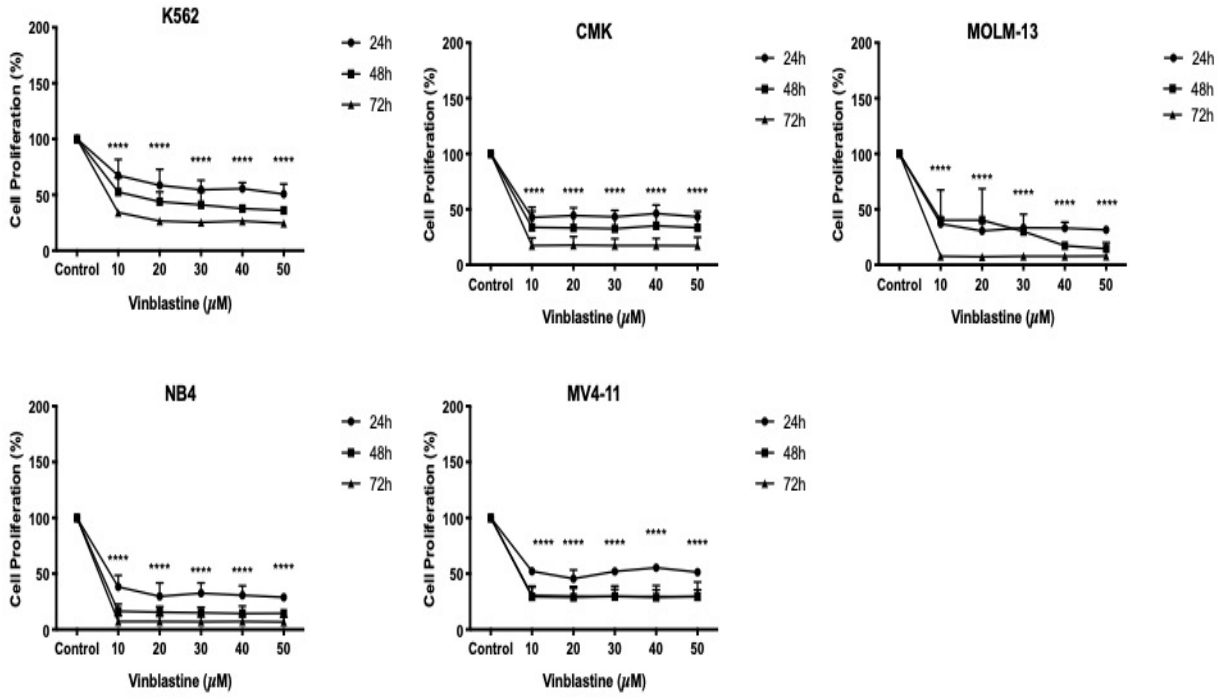


Figure 2.Vinblastine treatment leads to a decrease in the proliferation of AML cell line (CMK, MOLM-13, NB4 and MV4-11) at different time points (24, 48 and 72hrs).

K562, CML cell line was used as a control. The number of replicates was 3 in each experiment and the average of the replicates performed during each experiment were imported to GraphPad, which is 3 wells per treatment. The independent experiments were done two times (n=2). The statistical analyses and the comparisons of more than two groups were performed by 2-way ANOVA with Dunnett's multiple comparisons test and the significance of the test was evaluated as $p < 0.05$ by using GraphPad Prism 8. (ns=$P > 0.05$, * =$P \leq 0.05$, **= $P \leq 0.01$=, *** =$P \leq 0.001$, **** =$P \leq 0.0001$). All data are presented as mean±s.d.

To further confirm our vinblastine results and the effect of blocking the autophagosome-lysosome fusion, we used another inhibitor of the autophagosome-lysosome fusion, which is Nocodazole. Initially we started Nocodazole treatment with concentrations ranging 15-50 μM, which led to a significant decrease in the proliferation of MV4.11, NB4, CMK and MOLM-13, while K562 cell lines was a little bit more resistant (Figure 3). Due to the lethality of the above-mentioned dosages on the AML cell lines, we repeated the Nocodazole treatment with lower concentrations from 0.005 to 0.025 μM for MV4-11, 0.01 to 0.04 μM for K562 and 0.01 to 0.1 μM for CMK, MOLM-13 and NB4 (Figure 4). We found that these lower doses didn't affect much the proliferation of K562, NB4 and MV4-11 compared to CMK and MOLM-13 (Figure 4). One explanation why K562, CML cell line, was more resistant to autophagy inhibition is that high levels expression level of *BCR-ABL*, thus leading to a very active autophagy flux, which suggests the presence of mechanisms that allows the bypass of inhibition (Baquero et al., 2018). On the other hand, AML cells have shown a lower basal autophagy level, which makes them more sensitive compared to autophagy inhibition compared to K562 (Stankov et al., 2014). Our experiments suggest that the different AML cell lines respond differently to inhibition of the autophagosome-lysosome fusion and further investigation is required to understand the exact mechanisms that led to these differences.

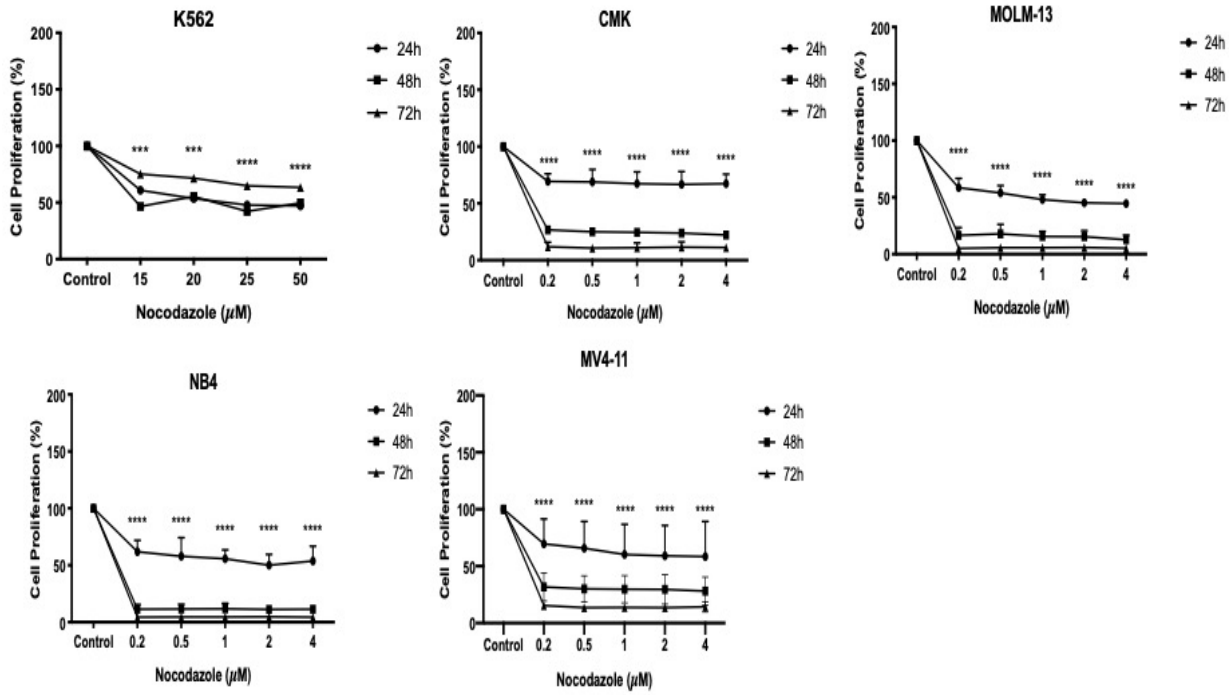


Figure 3. Nocodazole treatment leads to a decrease in the proliferation of AML cell line (CMK, MOLM-13, NB4 and MV4-11) at different time points (24, 48 and 72hrs).
K562, CML cell line was used as a control. The number of replicates was 3 in each experiment and the average of the replicates performed during each experiment were imported to GraphPad, which is 3 wells per treatment. The independent experiments were done two times for CMK, MOLM-13, NB4 and MV4-11(n=2) as for K562, the experiment was done once (n=1). The statistical analyses and the comparisons of more than two groups were performed by 2-way ANOVA with Dunnett's multiple comparisons test and the significance of the test was evaluated as p <0.05 by using GraphPad Prism 8. (ns=P > 0.05, * =P ≤ 0.05, **= P ≤ 0.01=, *** =P ≤ 0.001, **** =P ≤ 0.0001). All data are presented as mean±s.d.

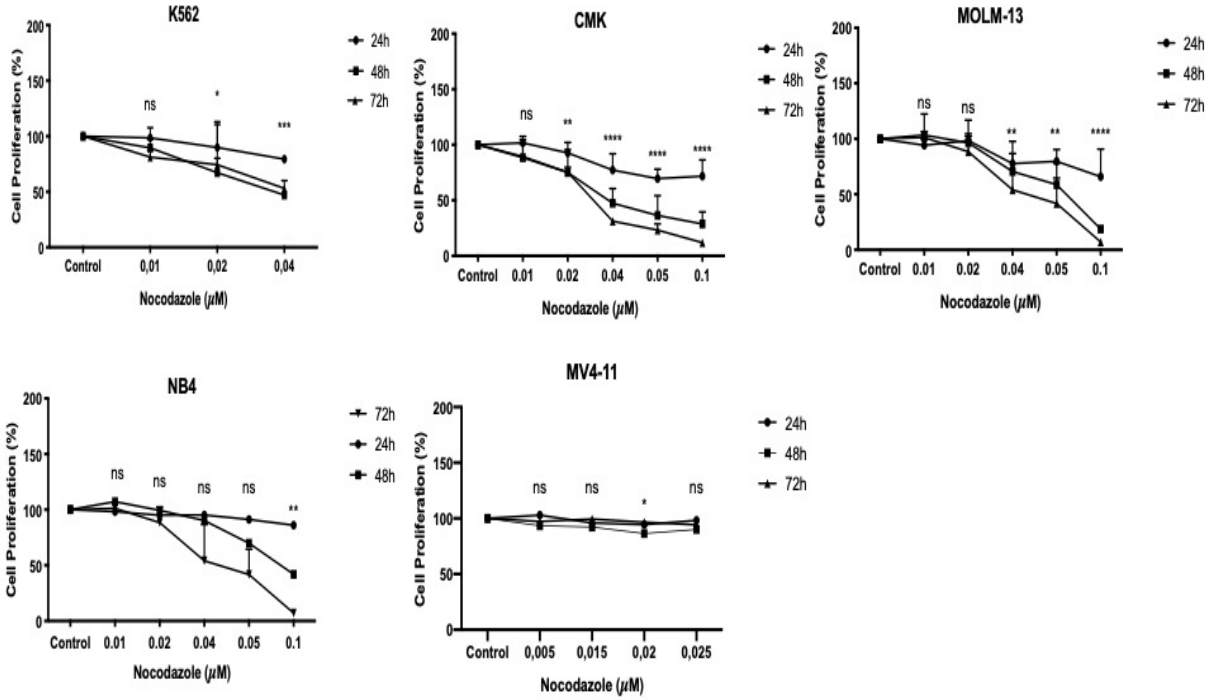


Figure 4. Lower doses of Nocodazole treatment leads to a decrease in the proliferation of CMK and MOLM-13 AML cell line compared to NB4 and MV4-11 at different time points (24, 48 and 72hrs).
K562, CML cell line was used as a control. The number of replicates was 3 in each experiment and the average of the replicates performed during each experiment were imported to GraphPad, which is 3 wells per treatment and the independent experiments were done three times for CMK (n=3), two times (n=2) for K562 and MOLM-13, once for NB4 and MV4-11 (n=1). The statistical analyses and the comparisons of more than two groups were performed by 2-way ANOVA with Dunnett's multiple comparisons test and the significance of the test was evaluated as p <0.05 by using GraphPad Prism 8. (ns=P > 0.05, * =P ≤ 0.05 , **= P ≤ 0.01=, *** =P ≤ 0.001, **** =P ≤ 0.0001). All data are presented as mean±s.d.

Then, we wanted to check the effect of the inhibition of the autophagolysosomal degradation. To do that, we used three different drugs; chloroquine (CQ) hydroxychloroquine (HQ) and $NH_4Cl$. CQ treatment also led to a significant decrease in the proliferation of AML cell lines (MV4.11, NB4, CMK, MOLM-13) and also to K562 cells line at different time points (Figure 5).

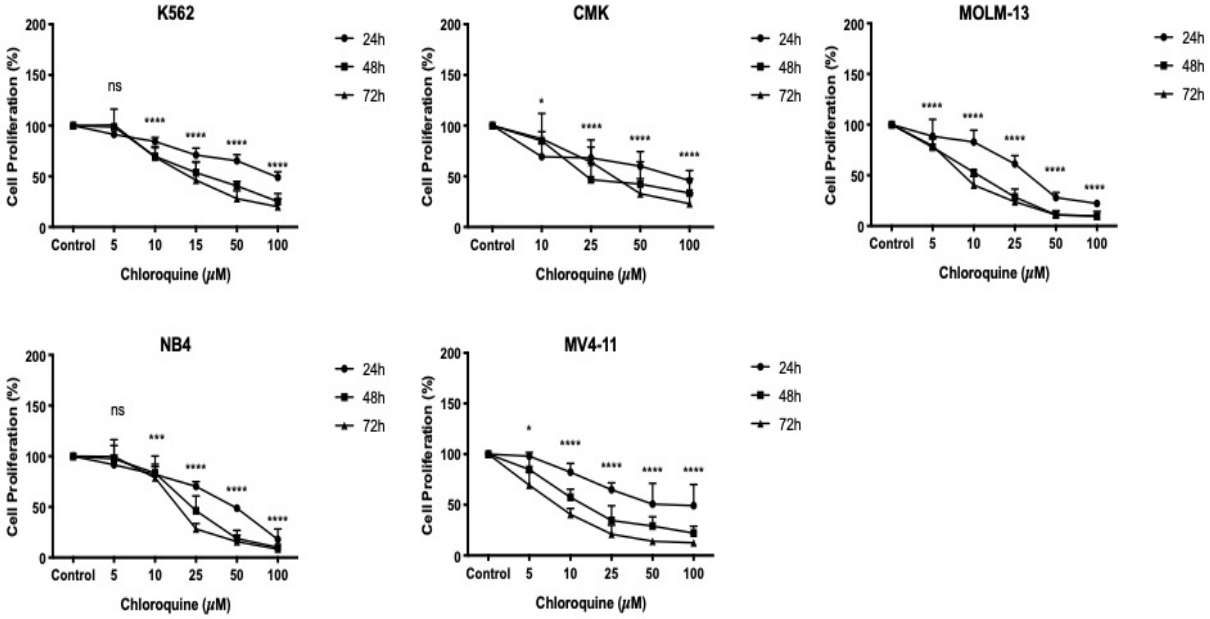


Figure 5. Chloroquine treatment leads to a decrease in the proliferation of AML cell line (CMK, MOLM-13, NB4 and MV4-11) at different time points (24, 48 and 72hrs).
K562, CML cell line was used as a control. The number of replicates was 3 in each experiment and the average of the replicates performed during each experiment were imported to GraphPad, which is 3 wells per treatment. The independent experiments were done three times (n=3). The statistical analyses and the comparisons of more than two groups were performed by 2-way ANOVA with Dunnett's multiple comparisons test and the significance of the test was evaluated as $p < 0.05$ by using GraphPad Prism 8. (ns=$P > 0.05$, * =$P \leq 0.05$ , **= $P \leq 0.01$=, *** =$P \leq 0.001$, **** =$P \leq 0.0001$). All data are presented as mean±s.d.

To further confirm that autophagy inhibition leads to the block in the proliferation of AML we treated the cells with Hydroxychloroquine. HQ treatment resulted in a significant decrease in the proliferation of AML cell lines (MV4.11, NB4, CMK, MOLM-13) and also to K562 cells line at different time points. Interestingly, CMK, NB4 and K562 sensitivity was at higher doses (20-100 μM) compared to other of AML cell lines. (Figure 6).

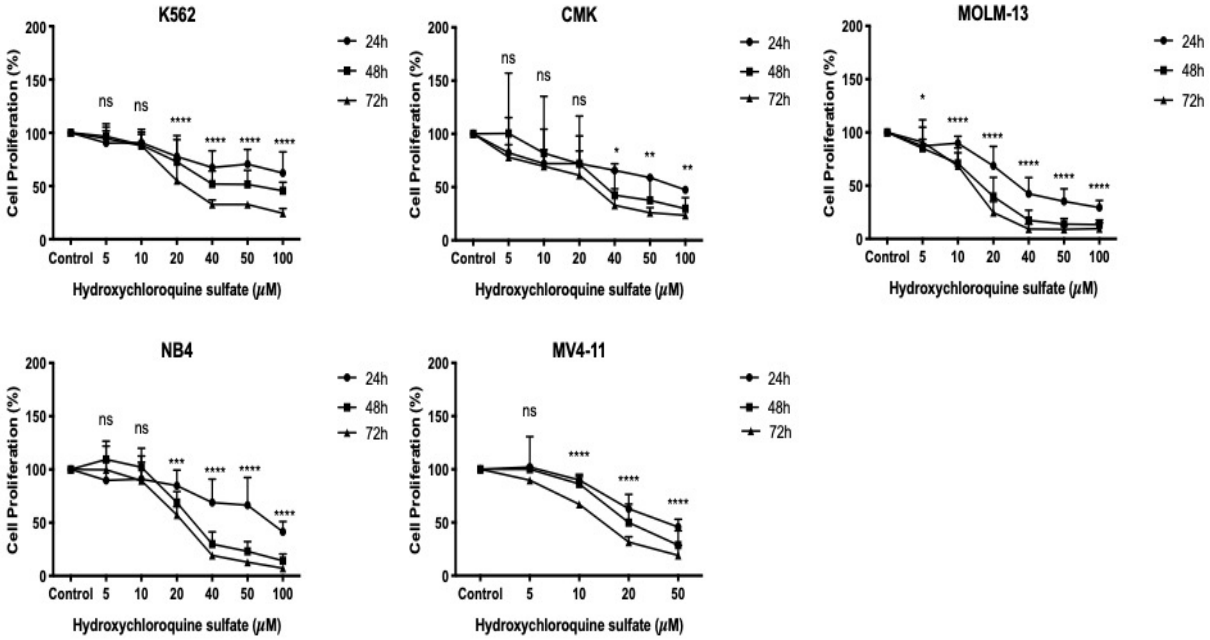


Figure 6. Hydroxychloroquine treatment leads to a decrease in the proliferation of AML cell line (CMK, MOLM-13, NB4 and MV4-11) at different time points (24, 48 and 72hrs).
K562, CML cell line was used as a control. The number of replicates was 3 in each experiment and the average of the replicates performed during each experiment were imported to GraphPad, which is 3 wells per treatment. The independent experiments were done five times for MOLM-13 (n=5), four times for K562 (n=4), three times for NB4 (n=3) and twice for CMK (n=2). The statistical analyses and the comparisons of more than two groups were performed by 2-way ANOVA with Dunnett's multiple comparisons test and the significance of the test was evaluated as $p < 0.05$ by using GraphPad Prism 8. (ns=$P > 0.05$, * =$P \leq 0.05$ , **= $P \leq 0.01$=, *** =$P \leq 0.001$, **** =$P \leq 0.0001$). All data are presented as mean±s.d.

$NH_4Cl$ showed the same effect on AML cell lines like CQ and HQ, where the proliferation of AML cell lines (MV4.11, NB4, CMK, MOLM-13). However, K562 was more resistant at lower concentration of $NH_4Cl$ treatment compared to the AML cell lines (Figure 7).

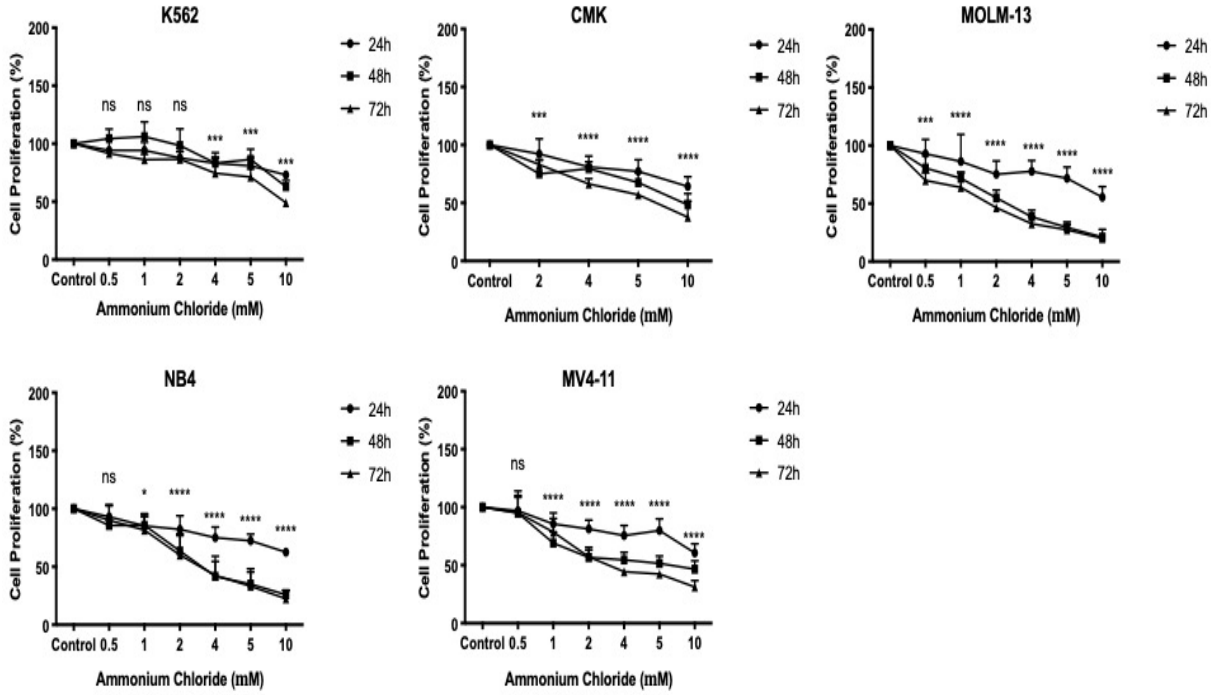


Figure 7. Ammonium Chloride ($NH_4Cl$) treatment leads to a decrease in the proliferation of AML cell line (CMK, MOLM-13, NB4 and MV4-11) at different time points (24, 48 and 72hrs). K562, CML cell line was used as a control. The number of replicates was 3 in each experiment and the average of the replicates performed during each experiment were imported to GraphPad, which is 3 wells per treatment . The independent experiments were done three times (n=3). The statistical analyses and the comparisons of more than two groups were performed by 2-way ANOVA with Dunnett's multiple comparisons test and the significance of the test was evaluated as $p < 0.05$ by using GraphPad Prism 8. (ns=$P > 0.05$, * =$P \leq 0.05$ , **= $P \leq 0.01$=, *** =$P \leq 0.001$, **** =$P \leq 0.0001$). All data are presented as mean±s.d.

Our data indicate AML cell lines are sensitive to autophagy inhibition no matter at which stage whether at the autophagosome-lysosome fusion or autophagolysosomal degradation. These data support data published by Stankov et al. (Stankov et al. 2014). However, the degree of sensitivity varies in the different cell lines at different doses and different time points of autophagy inhibition. In general, CMK, which is an AMKL cell line, appear to be sensitive to autophagy inhibition. This is due to the fact that, DS-AMKL cell lines and patient samples show a low basal level of autophagy due to the activation of the mTOR pathway by the aberrant insulin growth factor (IGF) signaling cascade (Stankov et al., 2014). NB4, MOLM13 and MV4-11 required higher doses and treatment time of CQ and $NH_4Cl$. This is due to the fact that they have a higher autophagic flux compared to CMK (Stankov et al., 2014). Thus, suppressing autophagy under a critical threshold allows the abrogation of leukemic growth. This highlights the importance of our study in trying to come up with a combination therapy of autophagy inhibitors in addition to hedgehog signaling pathway inhibitor, in the hope of accomplishing a synergistic effect that may provide a unique approach to target leukemic precursors in this disease. Till now our data is promising and in accordance with the existing literature, however,

we had some problems in the delivery of our GANT61 inhibitor, which prevented us from performing the all of the required experiments for this report.

### 4.3 The chemical activation of autophagy had no significant effect on the proliferation of the majority of AML cell lines.

To further study the effect of autophagy on AML cell lines (NB4, MOLM-13, MV4-11 and CMK) in comparison to other leukemia cell lines (K562), we treated the cells with autophagy activator, PP242, for different time points (24, 48 and 72 hrs). We have seen that the proliferation of NB4 and MOLM-13 cell lines were not affected by PP242 treatment after 24, 48 and 72 hrs in comparison to MV4-11 and K562 cell line, which were more sensitive to PP242 treatment. On the other hand, CMK, which has a low basal level of autophagy was sensitive to PP242 treatment at lower doses, however, at 0.05 μM these cells were not affected by PP242 treatment (Figure 8).

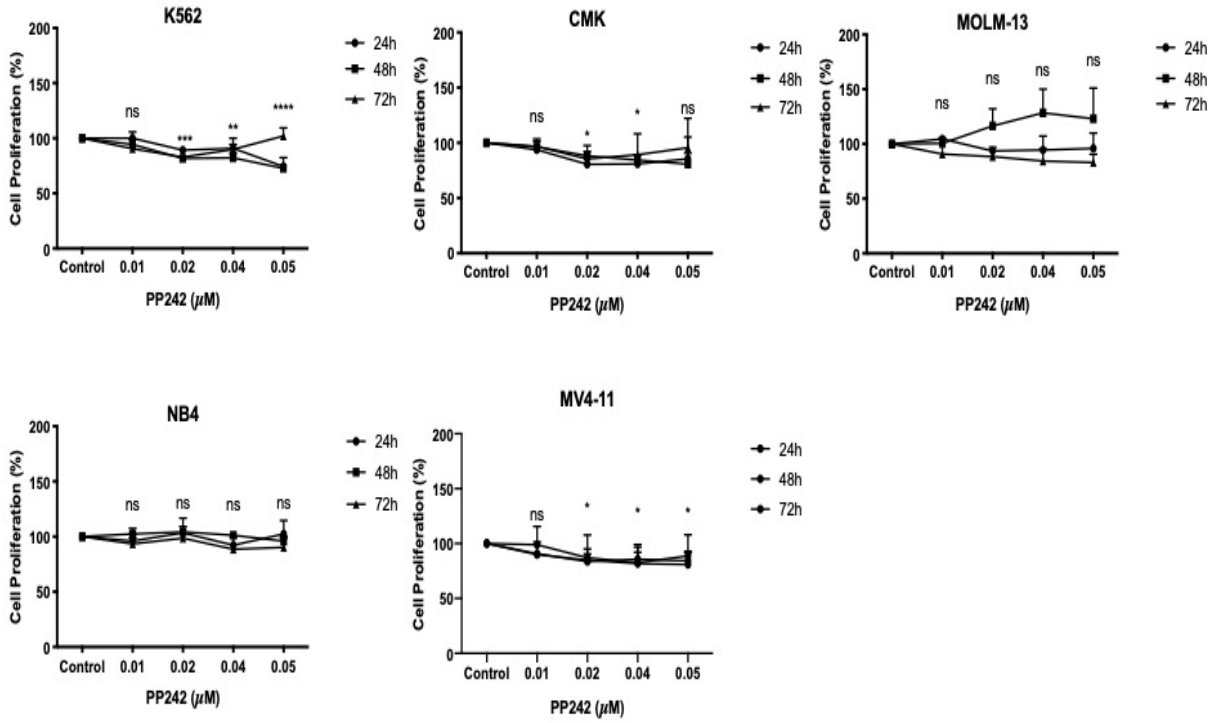


Figure 8. The effect of PP242 treatment on the proliferation of of AML cell line (CMK, MOLM-13, NB4 and MV4-11) at different time points (24, 48 and 72hrs).
K562, CML cell line was used as a control. The number of replicates was 3 in each experiment and the average of the replicates performed during each experiment were imported to GraphPad, which is 3 wells per treatment. The independent experiments were done three times (n=3). The statistical analyses and the comparisons of more than two groups were performed by 2-way ANOVA with Dunnett's multiple comparisons test and the significance of

the test was evaluated as p <0.05 by using GraphPad Prism 8. (ns=P > 0.05, * =P ≤ 0.05 , **= P ≤ 0.01=, *** P ≤ 0.001, **** =P ≤ 0.0001). All data are presented as mean±s.d.

Autophagy activation had different effects on the proliferation of AML cell lines this could be due to the fact that these cells have different basal autophagy levels. CMK was shown by Stankov et al. to have a low basal level of autophagy due to the activation of the IGF/IGF1R/PI3K/mTOR pathway (Klusmann et al., 2010; Stankov et al. 2014). In addition to that, MV4-11, which showed sensitivity to PP242 compared to other AML cell lines, is an *FLT3*-ITD positive cell line and recently a study that was presented at The American Society of Hematology (ASH) have shown that *FLT3*-ITD expression enhances autophagic flux in *FLT3*-ITD+ AML cells in a kinase-independent manner, and that increased autophagy contributes to TKI resistance of *FLT3*-ITD+ AML cells. Thus, a better understanding of autophagy is required in the context of AML, especially to understand what is the basal autophagy level that is required for the survival of the different AML cell lines from different cytogenetic backgrounds.

### 4.4. Autophagy inhibition leads to an increase in apoptotic cell death in CMK, MOLM-13, and MV4-11

To further confirm the results of the decrease in proliferation of AML cells after autophagy inhibition we performed Annexin V staining to detect the percentage of apoptotic cell death using the IC50 and IC20 values for 48hrs. MV4.11 showed a significant increase in the percentage of apoptotic cells compared to the control after treatment with CQ (10 and 50μM). Apoptotic population increased slightly after $NH_4Cl$ and HQ treatment (Figure 9).

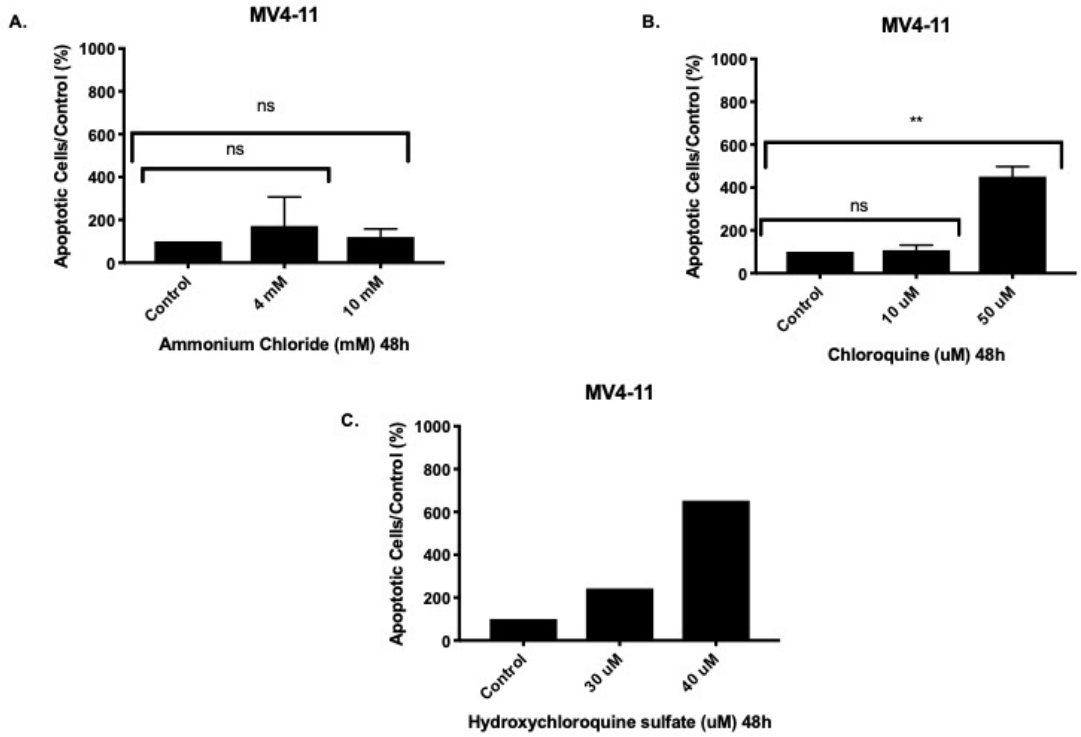


Figure 9. Autophagy inhibition using A. Ammonium chloride ($NH_4Cl$; 4mM and 10mM), B. Chloroquine (10 and 40 µM) and C. Hydroxychloroquine (30 and 40 µM) in MV4.11 cells for 48hrs leads to an increase in the percentage of apoptotic cells compared to the control.
The data was normalized to the control. The experiment was done twice (n=2) for $NH_4Cl$ and CQ and once for HQ. The number of replicates per experiment was done once. The significance of the test was evaluated as $p < 0.05$ by using GraphPad Prism 8. (ns=$P > 0.05$, * =$P \leq 0.05$ , **= $P \leq 0.01$=, *** $P \leq 0.001$, **** =$P \leq 0.0001$). All data are presented as mean±s.d.

NB4 cells also had more apoptotic cells compared to control especially after CQ (15 and 100 µM) and HQ treatment (20 and 40 µM). However, we have to repeat these experiments again (Figure 10 B and C). $NH_4Cl$ was didn't show a high increase in apoptotic cells in NB4 (Figure 10A) .

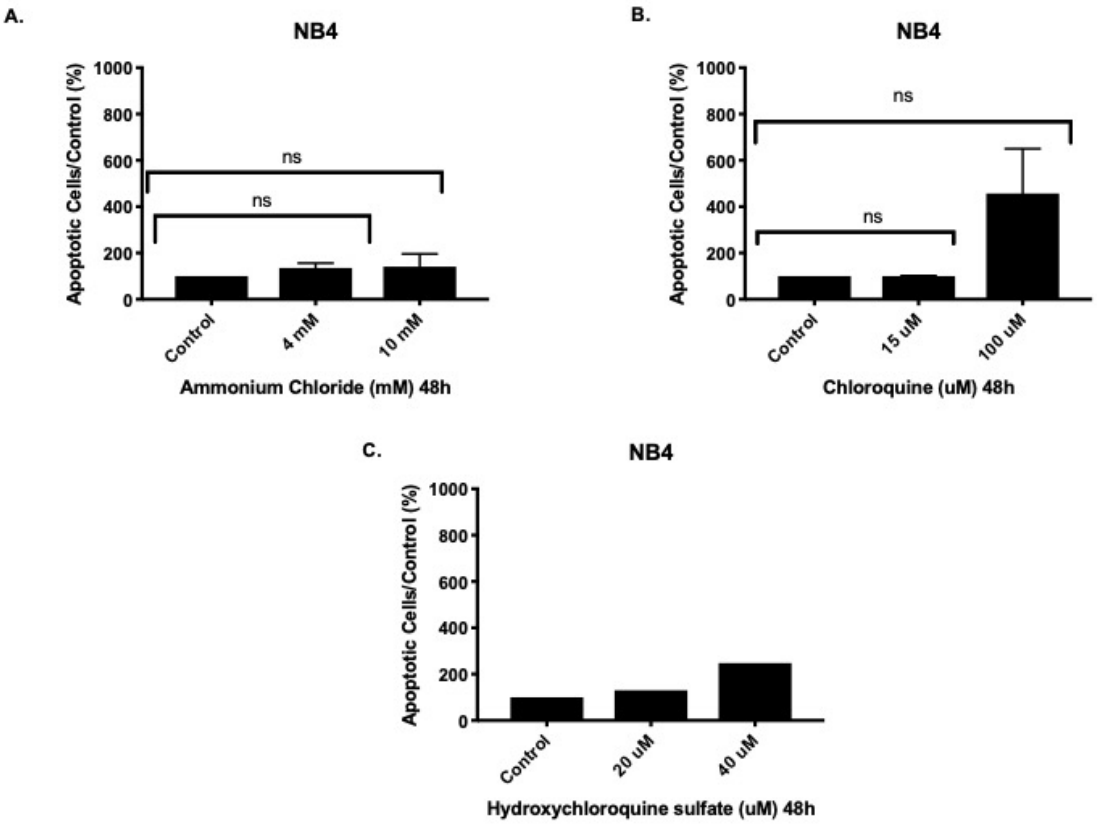


Figure 10. Autophagy inhibition using A. Ammonium chloride ($NH_4Cl$; 4mM and 10mM), B. Chloroquine (15 and 100 µM) and C. Hydroxychloroquine (20 and 40 µM) in NB4 cells for 48hrs leads to an increase in the percentage of apoptotic cells compared to the control.
The data was normalized to the control. The experiment was done twice times (n=2) for $NH_4Cl$ and CQ and once (n=1) for HQ. The number of replicates per experiment was done once. The significance of the test was evaluated as $p < 0.05$ by using GraphPad Prism 8. (ns=$P > 0.05$, * =$P \leq 0.05$ , **= $P \leq 0.01$=, *** $P \leq 0.001$, **** =$P \leq 0.0001$). All data are presented as mean±s.d.

For CMK cell lines, $NH_4Cl$ (10 and 20 mM) treatment had no effect on apoptosis but CQ (50 and 100 µM) treatment led to an increase in the percentage of apoptotic cells (Figure 11A and B).

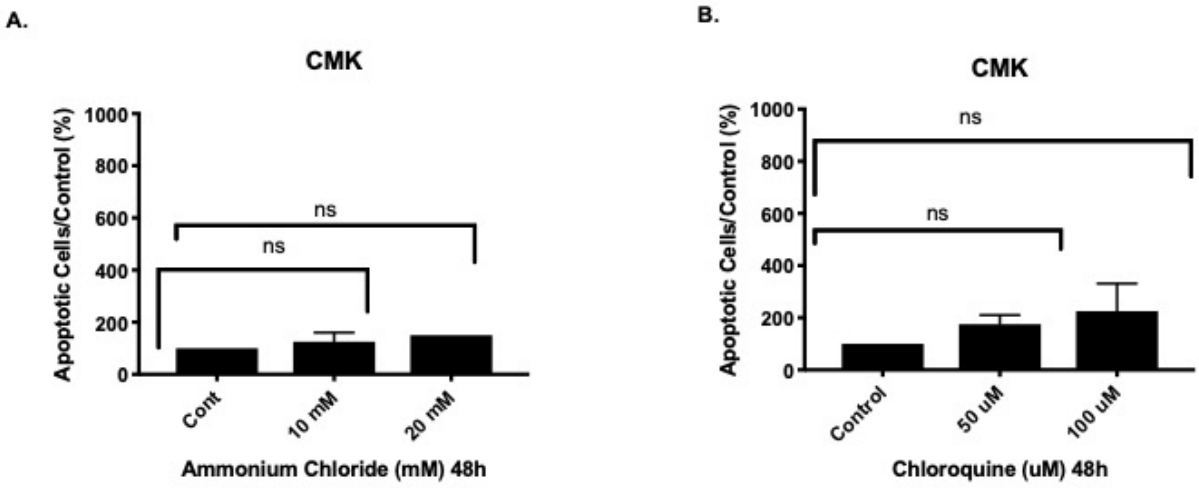


Figure 11. Autophagy inhibition using A. Ammonium chloride ($NH_4Cl$;10mM and 20mM) and B. Chloroquine (50 and 100μM) in CMK cells for 48hrs leads to an increase in the percentage of apoptotic cells compared to the control.
The data was normalized to the control. The experiment was done twice (n=2). The number of replicates per experiment was done once. The significance of the test was evaluated as p <0.05 by using GraphPad Prism 8. (ns=P > 0.05, * =P ≤ 0.05 , **= P ≤ 0.01=, *** P ≤ 0.001, **** =P ≤ 0.0001). All data are presented as mean±s.d.

For MOLM-13, there was an increase in the percentage of apoptotic cells compared to control after $NH_4Cl$ (2 and 10mM) and HQ (15 and 30 μM) treatment (Figure 12 A and C) and a significant increase after CQ treatment (30 μM) (Figure 12 B).

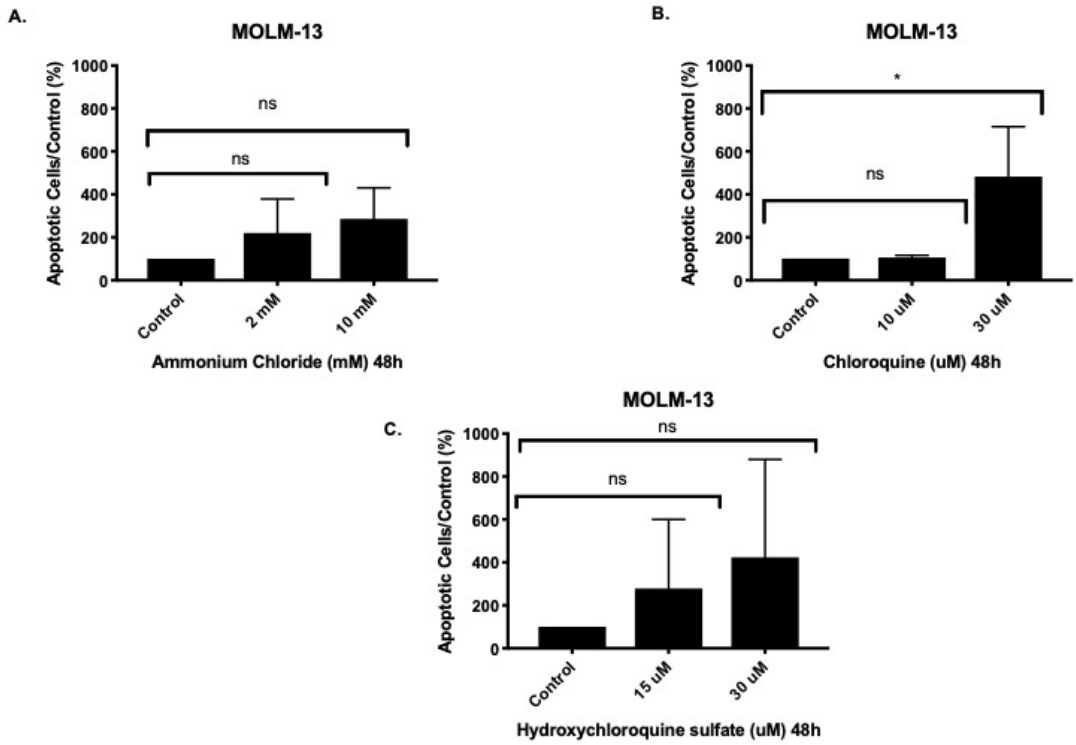


Figure 12. Autophagy inhibition using A. Ammonium chloride ($NH_4Cl$; 2mM and 10mM) and B. Chloroquine (10 and 30μM) and HQ (15 and 30μM) in MOLM-13 cells for 48hrs leads to an increase in the percentage of apoptotic cells compared to the control.
The data was normalized to the control. The experiment was done three times (n=3). The number of replicates per experiment was done once. The significance of the test was evaluated as p <0.05 by using GraphPad Prism 8. (ns=P > 0.05, * =P ≤ 0.05 , **= P ≤ 0.01=, *** P ≤ 0.001, **** =P ≤ 0.0001). All data are presented as mean±s.d.

K562 cells also had more apoptotic cells compared to control especially after $NH_4Cl$ (15 and 20 mM), CQ (15 and 100 μM) and HQ treatment (30 and 100 μM) (Figure 13). However, this experiment was done once and we need to repeat again to further confirm our data.

Depending on the data that we have obtained from the viability assay, we expected a higher percentage of apoptotic cells in AML treated cell lines in comparison with the control. We need to further confirm our data by checking molecular markers for apoptosis such as Poly (ADP-ribose) polymerase (PARP) cleavage and caspase 3. This induction of apoptosis after autophagy inhibition was seen in MOLM-13, CMK and K562 cells in multiple studies (Stankov et al. 2014; Folkert et al., 2017).

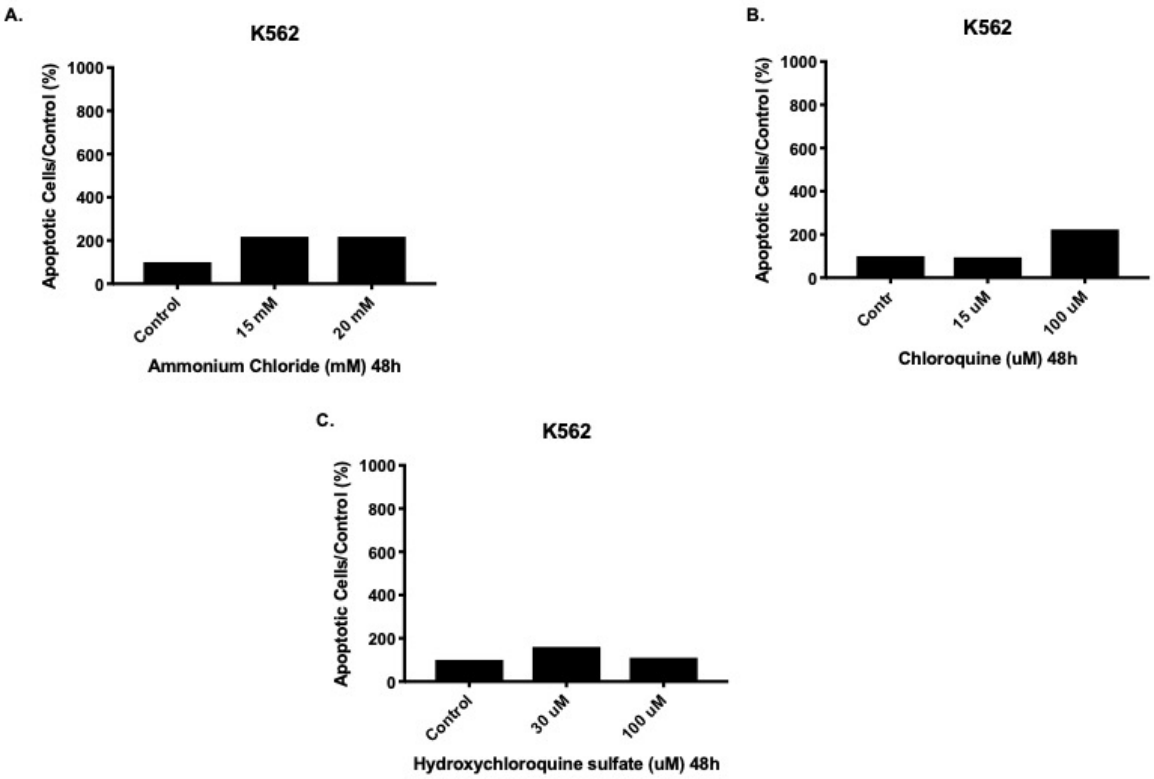


Figure 13. Autophagy inhibition using A. Ammonium chloride ($NH_4Cl$; 5mM and 10mM) and B. Chloroquine (15 and 100μM) and Hydroxychloroquine (30 and 100 μM) in K562 cells leads to an increase in the percentage of apoptotic cells compared to the control.
The data was normalized to the control. The experiment was done once (n=1).

### 4.5. The combination treatment of Autophagy blockers and hedgehog inhibitors led to an abrogation in the proliferation of MOLM-13 cell lines and to a lower extent in CMK cell lines.

As a first step to understand the interaction of HH and autophagic pathways in the context of AML and their effect on its pathogenesis, we performed a combinational-targeted therapy of different autophagy inhibitors in combination with GANT61, GLI1 inhibitor (Figure 14-15).
The results of this combination treatment will allow us to detect if there is any synergistic effect on the proliferation of AML cells. Both CMK and MOLM-13 cells were treated with GANT61 or Nocodazole alone and combination of GANT61 and Nocodazole for 48h. IC30 doses of Nocodazole for MOLM-13 (0.03 μM) and CMK (0.02 μM) and the IC20 (5 μM) and IC30 (6 μM) for GANT 61 were determined by cell proliferation assays (Figure 14A and 15A). When the CMK cells were treated with GANT61 or Nocodazole alone, the proliferation of cells decreased by around 20-30% of cells compared to DMSO control and water control. On the other hand, the combination treatment of GANT61 (IC30) and Nocodazole led to slight decrease in cell proliferation compared to GANT61 or Nocodazole alone and when comparing GANT61 IC30 or Nocodazole IC30 bars to the combination treatment of IC30 dosages of both GANT61.

However, GANT61 IC30 or Nocodazole IC20 treatment showed no effect on the proliferation of CMK. We can conclude that the combination treatment in this case has no synergistic effect (Figure 14 A). Similarly, in CMK cell lines, the combination treatment $NH_4Cl$ and GANT61 had no synergistic effect (Figure 14 B). In fact, interestingly, CMK cells treated with a combination of IC30 GANT61 and IC20 $NH_4Cl$, had an antagonistic effect leading to an increase in cell proliferation compared to single treatments (Figure 14B).

The combination treatment of Hedgehog and autophagy inhibitors on MOLM-13 cells was more promising compared to CMK cells. Upon comparing GANT61 IC20 and IC30 or Nocodazole IC30 bars to the combination treatment of both GANT61 and Nocodazole bars we can conclude that the combination treatment in this case is synergistic leading to a significant decrease in the proliferation of MOLM-13, AML cell line with an FLT3-ITD mutation (Figure 15A). This was also seen when we treated the MOLM-13 cells with GANT61 IC20 and IC30 or Chloroquine (CQ) IC30 (5μM) bars to the combination treatment of both GANT61 and CQ bars we can conclude that the combination treatment in this case is also synergistic leading to a block in the proliferation of these cells (Figure 15B).

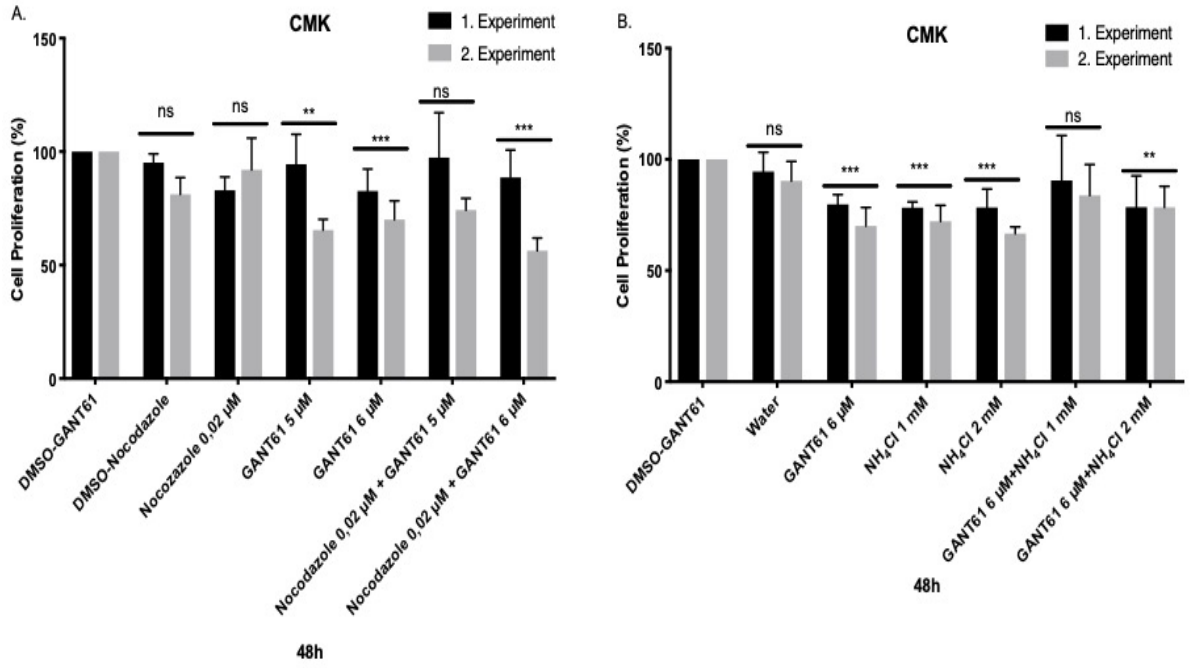


Figure 14. The effect of combination treatment of hedgehog and autophagy inhibitors on the proliferation of CMK cell line.
A. The proliferation of CMK cells was compared after the treatment with either IC20 dosages of GANT61 (5μM), IC30 dosages of GANT61(6μM), IC30 dosage of Nocodazole (0.02μM), or their combinations. B. The proliferation of CMK cells was compared after the treatment with either IC20 dosages of $NH_4Cl$ (1mM), IC30 dosage of $NH_4Cl$ (2mM) and IC30 dosages of GANT61 (6μM), , or their combinations. The number of replicates was 3 in each experiment, which is 3 wells per treatment and the independent experiments were done two times (n=2). The statistical analyses and the comparisons of more than two groups were performed by 2-way ANOVA with Dunnett's multiple comparisons test and the significance of the test was

evaluated as p <0.05 by using GraphPad Prism 8. (ns=P > 0.05, * =P ≤ 0.05 , **= P ≤ 0.01=, *** P ≤ 0.001, **** =P ≤ 0.0001). All data are presented as mean±s.d.

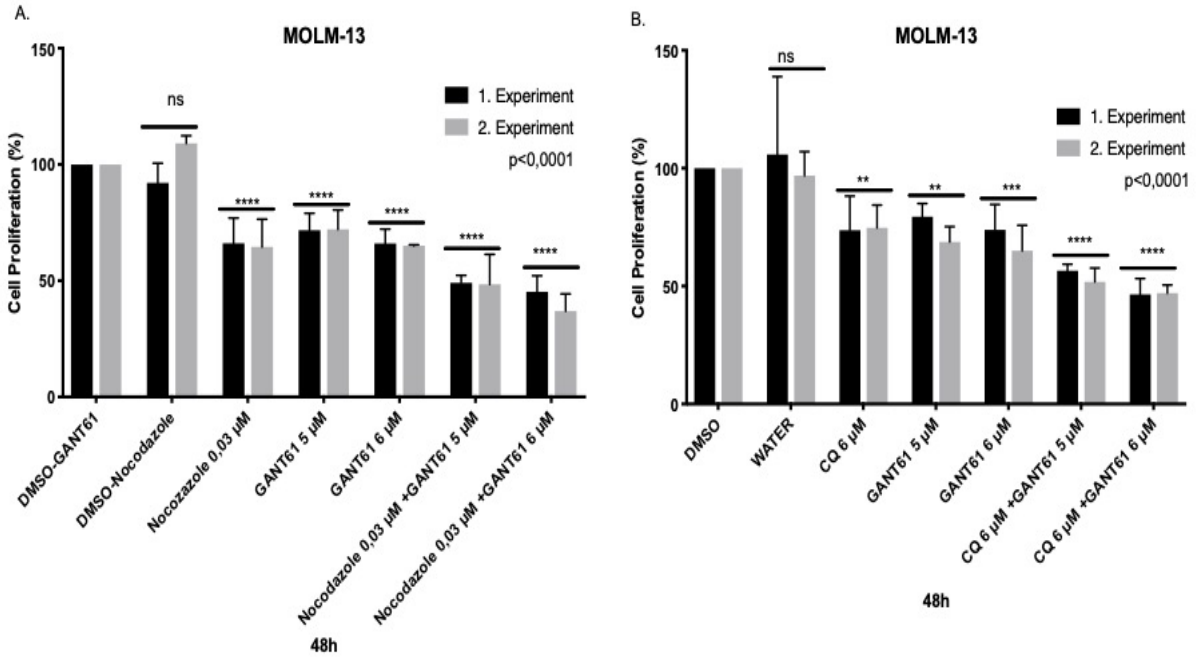


Figure 15. The effect of combination treatment of hedgehog and autophagy inhibitors on the proliferation of MOLM-13 AML cell line.
A. The proliferation of MOLM-13 cells was compared after the treatment with either IC20 dosages of GANT61 (5µM), IC30 dosages of GANT61(6µM), IC30 dosage of Nocodazole (0.03µM), or their combinations. B. The proliferation of MOLM-13 cells was compared after the treatment with either IC20 dosages of GANT61 (5µM), IC30 dosages of GANT61, IC30 dosage of Chloroquine (CQ) (5µM), or their combinations. The number of replicates was 3 in each experiment, which is 3 wells per treatment and the independent experiments were done two times (n=2). The statistical analyses and the comparisons of more than two groups were performed by 2-way ANOVA with Dunnett's multiple comparisons test and the significance of the test was evaluated as p <0.05 by using GraphPad Prism 8. (ns=P > 0.05, * =P ≤ 0.05 , **= P ≤ 0.01=, *** P ≤ 0.001, **** =P ≤ 0.0001). All data are presented as mean±s.d.

These findings fit our hypothesis that the response to combination treatment is highly dependent on cellular context. The combination treatment was more effective on MOLM-13, *FLT3*-ITD AML, cell compared to CMK, AMKL cells. This could be due to the fact that CMK cells has a low basal level of autophagy due to the activation of the IGF/IGF1R/PI3K/mTOR pathway which promotes its survival and high resistance to therapy (Klusmann et al., 2010; Stankov et al. 2014). It is also known that blocking the hedgehog pathway leads to the activation of autophagy (Wang et al. 2014; Xu et al., 2014; Zeng et al., 2015; Wang et al., 2016; Wang et al. 2018a; Wang et al. 2018b). It could be that this activation in the case of CMK

promotes its survival and diminishes the effect of autophagy inhibition. One explanation could be that the activation of autophagy increases the basal autophagy level of these cells above which autophagy blockers had no effect anymore since CMK cells already requires a really low basal level of autophagy for their survival. On the other hand, MOLM-13 cells were sensitive to the combination treatment. This synergistic effect of HH and autophagy inhibitors was seen in Chronic myeloid Leukemia, neuroblastoma and multiple types of cancer (Wang et al., 2013; Wang et al., 2014; Zeng et al., 2015; Wang et al., 2016; Wang et al., 2017; Wang et al, 2018a; Wang et al 2018b). However, further experiments are required to assess the effect of this combination treatment on other AML cell lines from different genetic backgrounds. For that we wanted to check the other AML cell lines but we had some problems in the delivery of our GANT61 inhibitor, which prevented us from performing these experiments.

### 4.6. Understanding the molecular mechanisms of hedgehog and autophagy inhibition.

In order to understand the effect of autophagy and hedgehog treatment, we sought to look at the molecular markers of autophagy. To do that we performed western blot assays on whole cell lysates. Firstly, we treated our different AML cell lines (MOLM-13, NB4, and CMK) and CML cell line (K562) with different autophagy modulators for 48 hrs. Inhibition of autophagy was achieved as follows; for the autophagosome-lysosome fusion we used nocodazole, while for the autophagolysosomal degradation we used on of the following: $NH_4Cl$, chloroquine (CQ) or hydroxychloroquine (HQ).

We started by checking the effect of the inhibition of the autophagolysosomal degradation on the expression of expression of the autophagy markers such as the microtubule-associated protein light chain 3 (LC3) I conversion to LC3-I in MOLM-13, NB4 and CMK AML cell line in addition to its expression in K562 cells. We found that upon $NH_4Cl$ treatment, MOLM-13 cells experienced an accumulation of LC3II, this is coinciding with the block of the fusion of the autophagosome to the lysosome, thus, inhibiting this step of autophagy due to $NH_4Cl$ treatment (Figure 16C-D and Figure 17A). This was also detected in NB4 (Figure 19 A-B) and CMK (Figure 20) cells but not in K562 (Figure18 C-D), where the expression of LC3I more elevated compared to LC3II, which suggests that the early activation of autophagy didn't occur and that their was an inhibition of autophagy during the early step which prevented the lipidation and conversion of LC3I to LC3II. Interestingly, after $NH_4Cl$ treatment for 48hrs, AKT (our antibody detects pan-AKT, i.e the different family of AKT protein, which are AKT1, AKT2 and AKT3), which is a main regulator of the PI3/AKT/mTOR pathway and an inhibitor of autophagy if activated, showed a decrease in expression in MOLM13 (Figure 17-18), NB4 (Figure 19 A-B)

and CMK (Figure 20) compared to K562, which showed an increase in AKT expression this correlates with the increase in LC3BI that we discussed before (Figure 18C-D) (Liu et al., 2019; Ren et al., 2019) . However, to be able to assess our AKT expression data objectively, we need to check the expression of phosphorylated AKT (p-AKT), which is the active version of the protein. A study done by Merhi et al. have studied the effect of $NH_4Cl$ on mTORC1 and mTORC2 and have found that upon treating multiple cancer cells with $NH_4Cl$, there was an increase in p-AKT and no change in the total AKT expression (Merhi et al. 2017). This indicates the importance of detecting the p-AKT in our cell lines. However, due to the tight budget we were not able to purchase this antibody.

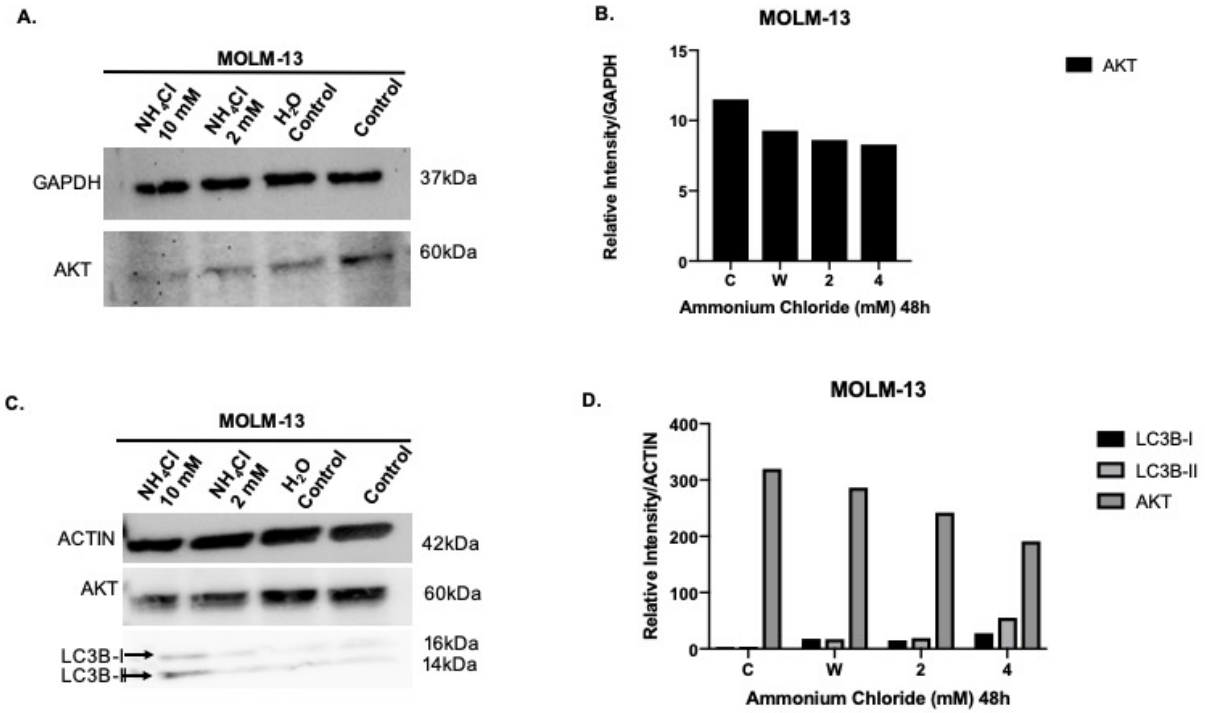


Figure 16. The expression of autophagy markers after ammonium chloride ($NH_4Cl$) treatment for 48hrs in MOLM 13 cells.
A. Expression of AKT and GAPDH (internal loading control) in untreated control, water control (vehicle), 2mM and 10 mM $NH_4Cl$. B. The quantification of AKT expression/GAPDH (Densitometric graph) in untreated control, water control (vehicle), 2mM and 10 mM $NH_4Cl$. C. Expression of AKT, LCBI/II and ACTIN (internal loading control) in untreated control, water control (vehicle), 2mM and 10 mM $NH_4Cl$. D. The quantification of AKT expression/ACTIN, LC3I/AKTIN and LC3BII/ACTIN (Densitometric graph) in untreated control, water control (vehicle), 2mM and 10 mM $NH_4Cl$.

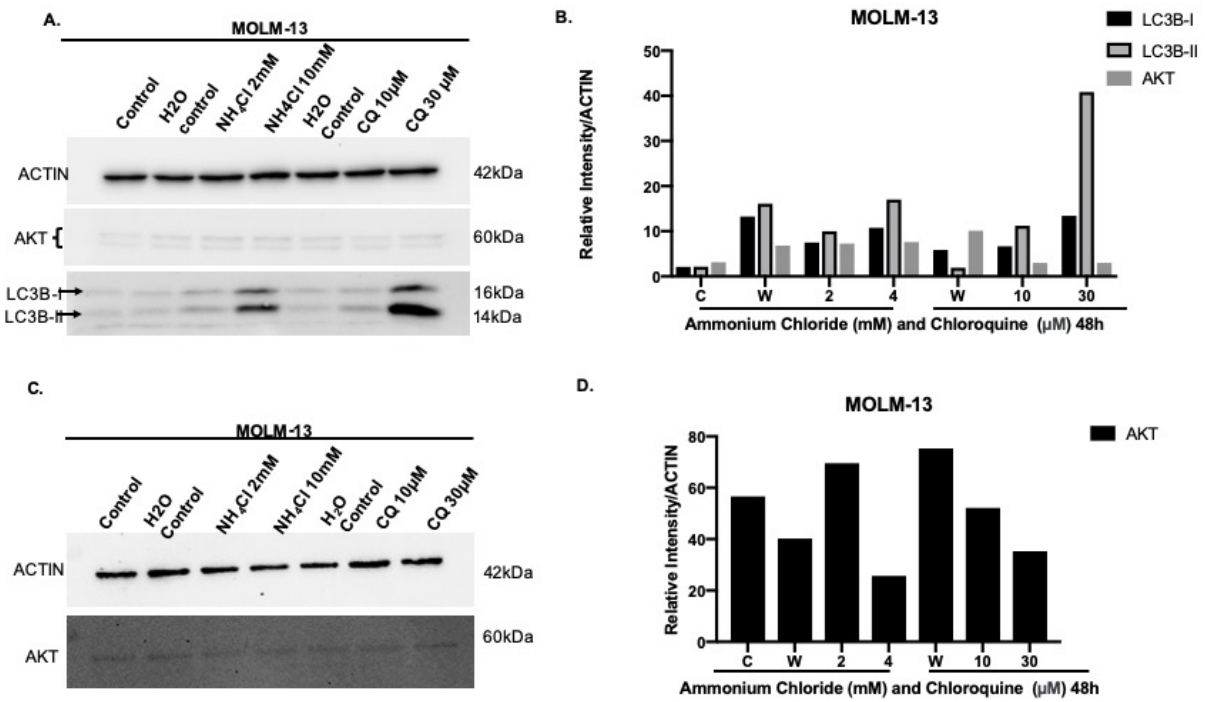


Figure 17. The expression of autophagy markers after ammonium chloride ($NH_4Cl$) and chloroquine (CQ) treatment for 48hrs in MOLM 13 cells.

A. Expression of AKT, LC3BI, LC3BII, and ACTIN (internal loading control) in untreated control, water control (vehicle for $NH_4Cl$), $NH_4Cl$ (2mM and 10 mM), water control (vehicle for CQ) and CQ (10 and 30 µM). B. The quantification of AKT/ACTIN, LC3I/ACTIN and LC3BII/ACTIN (Densitometric graph) in untreated control, water control (vehicle for $NH_4Cl$), 2mM and 10 mM $NH_4Cl$, water control (vehicle for CQ), 10 and 30 µM CQ. C. Expression of AKT and ACTIN (internal loading control) in untreated control, water control (vehicle), 2mM and 10 mM $NH_4Cl$. D. The quantification of AKT/ACTIN (Densitometric graph) in untreated control, water control (vehicle for $NH_4Cl$), $NH_4Cl$ (2mM and 10 mM), water control (vehicle for CQ) and CQ (10 and 30 µM).

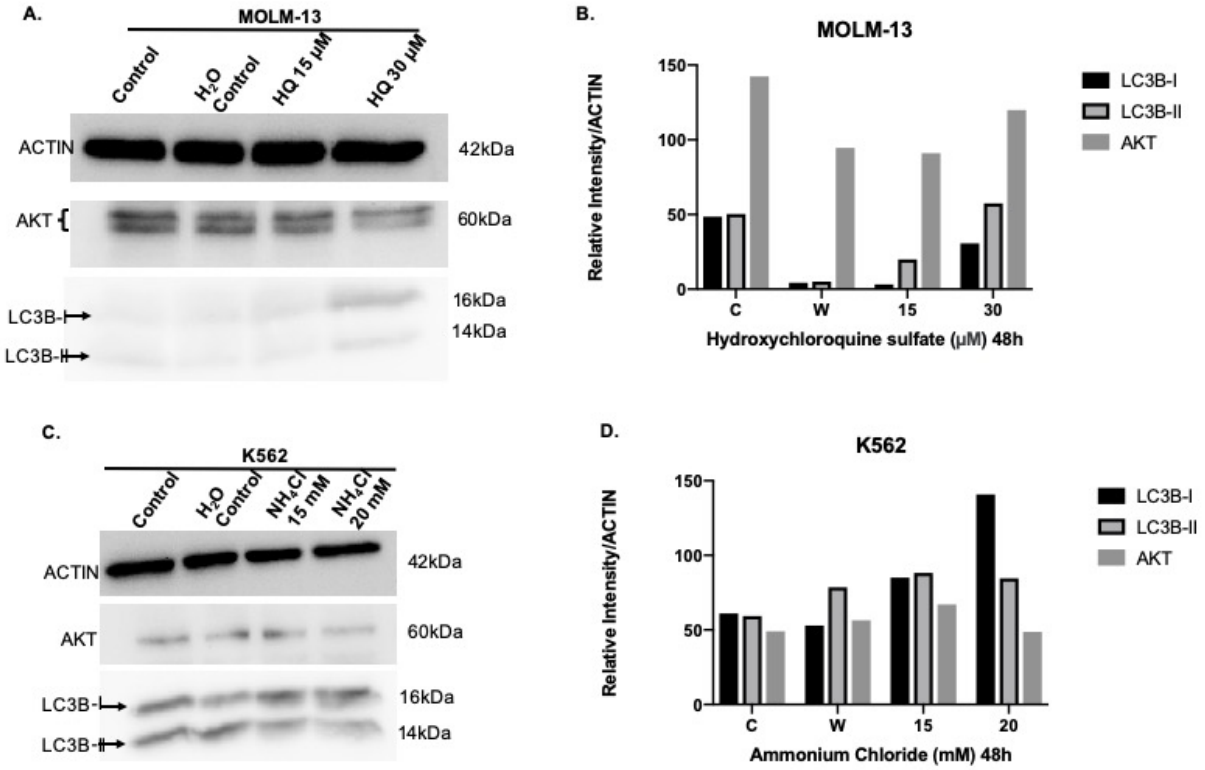


Figure 18. The expression of autophagy markers after autophagy inhibition treatment for 48hrs in MOLM-13 and K562 cells.

A. Expression of AKT, LC3BI, LC3BII, and ACTIN (internal loading control) in MOLM-13 untreated control, water control (vehicle for HQ), Hydroxychloroquine (HQ) (15 µM and 30 µM). B. The quantification of AKT/ACTIN, LC3I/ACTIN and LC3BII/ACTIN (Densitometric graph) in MOLM-13 untreated control, water control (vehicle for HQ), Hydroxychloroquine (HQ) (15 µM and 30 µM). C. Expression of AKT, LC3BI, LC3BII, and ACTIN (internal loading control) in K562 untreated control, water control (vehicle for $NH_4Cl$) and NH4Cl (15mM and 20mM). B. The quantification of AKT/ACTIN, LC3I/ACTIN and LC3BII/ACTIN (Densitometric graph) in K562 untreated control, water control (vehicle for $NH_4Cl$) and NH4Cl (15mM and 20mM).

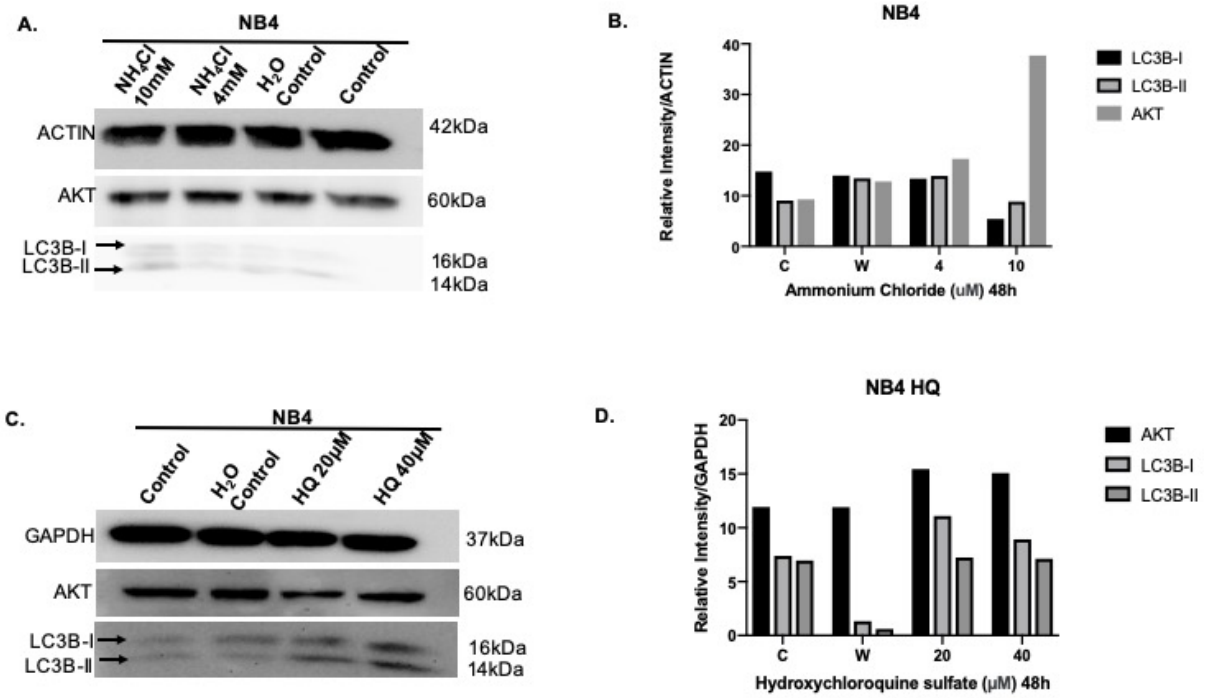


Figure 19. The expression of autophagy markers after autophagy inhibition treatment for 48hrs in NB4 cells.

A. Expression of AKT and ACTIN (internal loading control) in untreated control, water control (vehicle for $NH_4Cl$), $NH_4Cl$ (4mM and 10mM). B. The quantification of AKTI/ACTIN, AKTII/ACTIN, LC3I/ACTIN and LC3BII/ACTIN (Densitometric graph) in untreated control, water control (vehicle for $NH_4Cl$), $NH_4Cl$ (4mM and 10mM). C. Expression of AKT, LC3BI, LC3BII, and GAPDH (internal loading control) in untreated control, water control (vehicle for HQ) and HQ (20µM and 40µM). B. The quantification of AKT/GAPDH, LC3I/GAPDH and LC3BII/GAPDH (Densitometric graph) in untreated control, water control (vehicle for HQ) and HQ (20µM and 40µM)

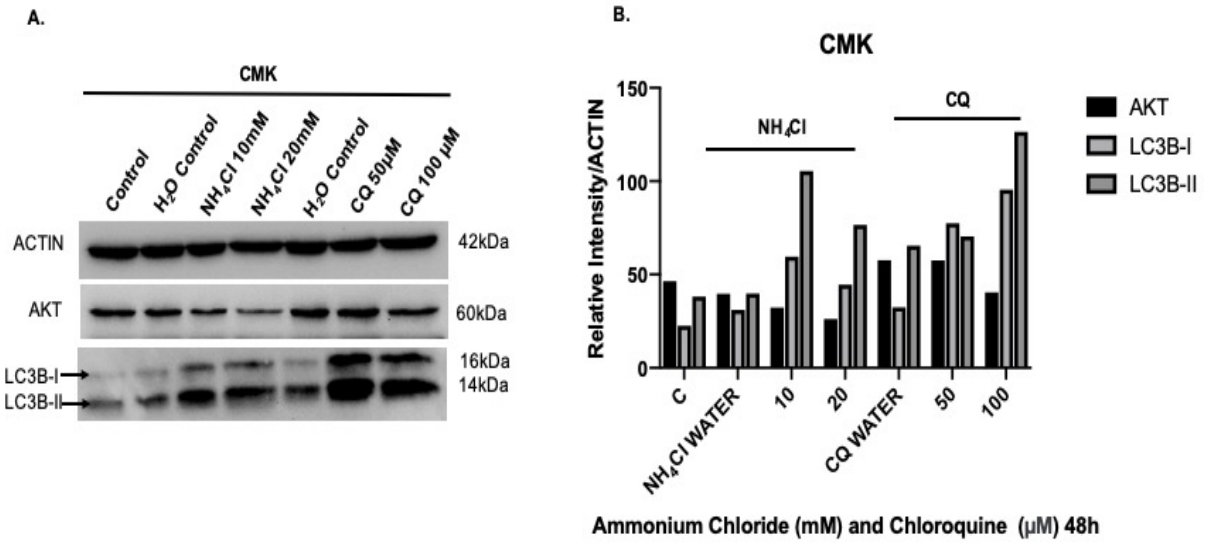


Figure 20. The expression of autophagy markers after autophagy inhibition treatment for 48hrs in CMK cells.
A. Expression of AKT and ACTIN (internal loading control) in untreated control, water control (vehicle for $NH_4Cl$), $NH_4Cl$ (10mM and 20mM). B. The quantification of AKT/ACTIN, LC3I/ACTIN and LC3BII/ACTIN (Densitometric graph) in untreated control, water control (vehicle for $NH_4Cl$), $NH_4Cl$ (4mM and 10mM). C. Expression of AKT, LC3BI, LC3BII, and ACTIN (internal loading control) in untreated control, water control (vehicle for CQ) and CQ (50µM and 100µM). B. The quantification of AKT/ACTIN, LC3I/ACTIN and LC3BII/ACTIN (Densitometric graph) in untreated control, water control (vehicle for CQ) and CQ (50µM and 100µM)

To further confirm the effects of the inhibition of autophagolysosomal degradation step of autophagy we used two other inhibitors of this process, which are chloroquine (CQ) and hydroxychloroquine (HQ). CQ treatment in MOLM-13 and CMK showed a similar trend to $NH_4Cl$ treatment. We detected a decrease in AKT expression in MOLM-13 and CMK (Figure17C-D, Figure 20) and an increase in LC3I to LC3II conversion in both cell lines (Figure 17A-B, Figure 20). This increase in LC3BII is due to the fact that CQ prevents autophagosome-lysosome fusion and thus blocks degradation process of autophagy (Tong et al. 2012). This was also shown in CML cells after treatment with CQ (Tong et al., 2012; Song et al., 2015).
Also, HQ treatment led to an accumulation in LC3I/LC3II in MOLM-13 (Figure18A-B) and NB4 (Figure 19C-D) cell lines and only in NB4 an increase in AKT levels (Figure 19C-D) compared to water control. This was also seen in some AML cell lines and resistant myeloid leukemias after HQ treatment (Kim et al., 2015; Folkerts et al., 2017). We have also tried antibodies against other autophagic proteins such as ULK-1 and ATG5 but unfortunately the antibodies did not work and the budget was not enough to purchase new ones.

After confirming the block in autophagy after treatment with different autophagy modulators, we subjected MOLM-13 and CMK AML cell lines to combination treatment of GANT61, HH inhibitor, along with either inhibitors targeting the autophagosome-lysosome fusion (nocodazole) or the autophagolysosomal degradation ($NH_4Cl$ or CQ) for 48 hrs. We used the same IC values that we have used for the proliferation assays; IC20 (5μM) or 30 (6μM) for GANT61, IC 30 (0.02μM or 0.03μM for CMK and MOLM-13, respectively) for Nocodazole, IC30 (6μM for MOLM-13) for CQ and IC20 (1mM for CMK) or IC30 (2mM for CMK) $NH_4Cl$. After that we performed western blot analysis on the whole cell lysates of MOLM-13 and CMK to detect the protein expression of the autophagic marker LC3I/II and the expression of AKT.

In MOLM-13 cells, nocodazole or GANT61 treatment alone didn't cause a change in the expression of AKT. However, the combination of 0.02μM Nocodazole and 6μM GANT61 led to an increase in AKT expression according to the densitometric analysis compared to single treatments and to the control (Figure 21 A-B). As expected, Nocodazole decreased the expression of LC3I/II conversion compared to the control and the combination of Nocodazole with the low dosage of GANT61 resulted in a decrease in the expression of LC3I/II compared to GANT61 treatment alone. However, Nocodazole could not block the induction of autophagy that is triggered by high dosage GANT61 treatment and this is reflected by an increase in the expression of LC3I and LC3II as shown by the densitometric analysis (Figure 21 A-B). It is known that HH pathway inhibits autophagy and the inhibition of the HH pathway by GANT61 leads to an activation of autophagy (Xu et al., 2014; Wang et al., 2018).

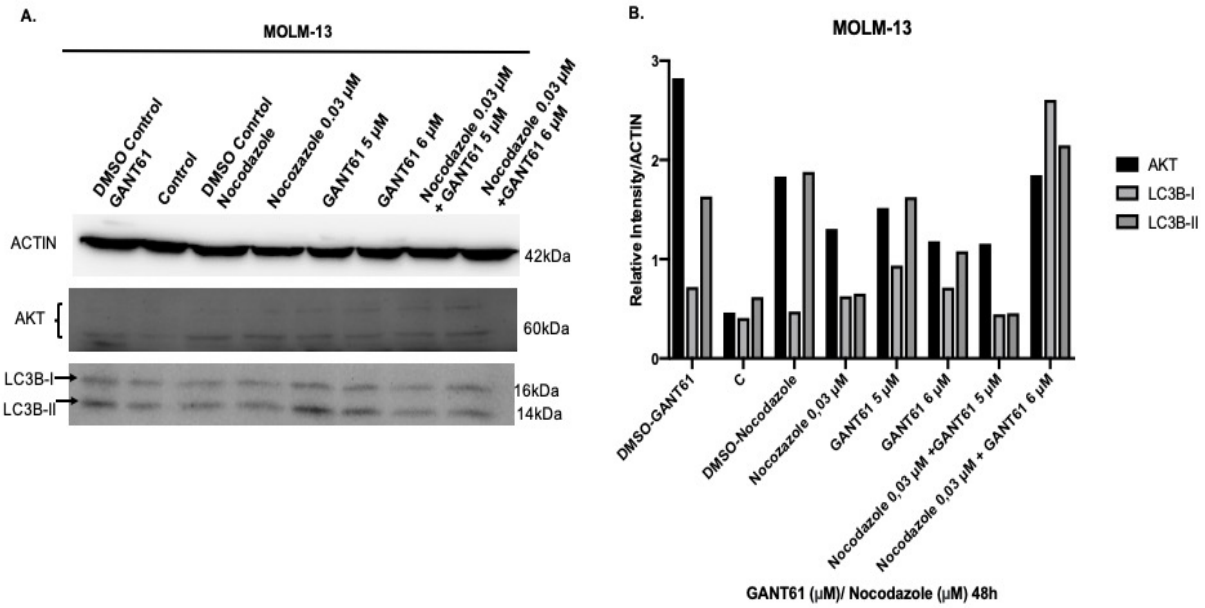


Figure 21. The expression of autophagy markers after autophagy and Hedgehog inhibition for 48hrs in MOLM-13 cells.
A. Expression of AKT, LC3I, LC3II and ACTIN (internal loading control) in untreated control, DMSO control (vehicle for GANT61 and Nocodazole), Nocodazole (0.03 µM), GANT61 (5 or 6 µM) and two different combinations of GANT61 and Nocodazole. B. The densitometric analysis of AKT/ACTIN, LC3I/ACTIN and LC3BII/ACTIN in in untreated control, DMSO control (vehicle for GANT61 and Nocodazole), Nocodazole (0.03 µM), GANT61 (5 or 6 µM) and two different combinations of GANT61 and Nocodazole.

Interestingly, when MOLM-13 cells were treated with either CQ or GANT61 alone or with the combination of CQ and 5 µM of GANT61 there was no change in AKT protein expression (Figure 22 A-B). However, when MOLM-13 cells were treated with CQ and 6 µM GANT61, densitometric analysis showed an increase in AKT expression. Further experiments are required to understand the exact mechanisms behind that increase especially that p-AKT is a known negative regulator of autophagy, thus, we have to check the expression of p-AKT before we come up with any decisive conclusions.

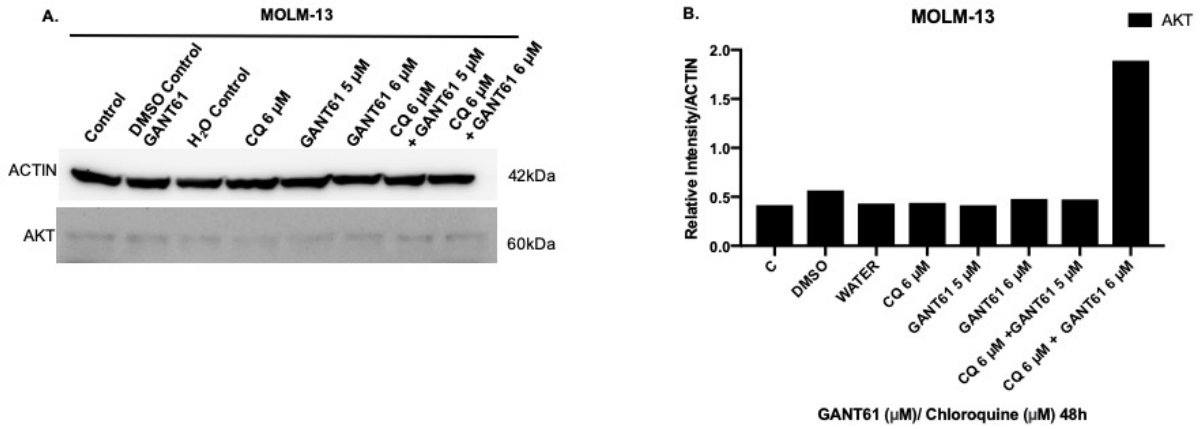


Figure 22. The expression of autophagy markers after autophagy and Hedgehog inhibition for 48hrs in MOLM-13 cells.
A. Expression of AKT and ACTIN (internal loading control) in untreated control, DMSO control (vehicle for GANT61), Water control (vehicle for CQ), CQ (6 µM), GANT61 (5 or 6 µM) and two different combinations of GANT61 and CQ. B. The densitometric analysis of AKT/ACTIN in untreated control, DMSO control (vehicle for GANT61), Water control (vehicle for CQ), CQ (6 µM), GANT61 (5 or 6 µM) and two different combinations of GANT61 and CQ.

In the context of CMK cell lines, similar to MOLM-13 there was no change in the level of AKT in single treatments of Nocodazole or GANT61 alone (Figure 23 A-B). However, we started to detect an increase combination of 0.02µM Nocodazole with 5µM GANT and a much higher increase when we treated the cells with 0.02µM Nocodazole with 6µM GANT (Figure 23A-B). However, we didn't detect a high induction in LC3I/II, which suggests that nocodazole treatment in the context of CMK blocks the autophagy that is induced by GANT61 treatment and this correlated to the low sensitivity that we have detect by CMK to the combination treatment (Figure 14). Additionally, we have treated CMK cells with a combination of GANT61 and the inhibitor of the autophagolysosomal degradation step of autophagy, $NH_4Cl$. We found that after $NH_4Cl$ and GANT61 single treatments, there was a decrease in AKT protein expression as shown by the densitometric analysis. However, that decrease was abrogated when we treated the cells with 2mM $NH_4Cl$ and 6µM GANT61 (Figure 24 A-B). Also, we detected an increase in the level of LC3II compared to the DMSO control. However, this increase was blocked when we treated the cells with the combination treatment especially at low doses of NH4Cl (1mM) with 6µM GANT61.

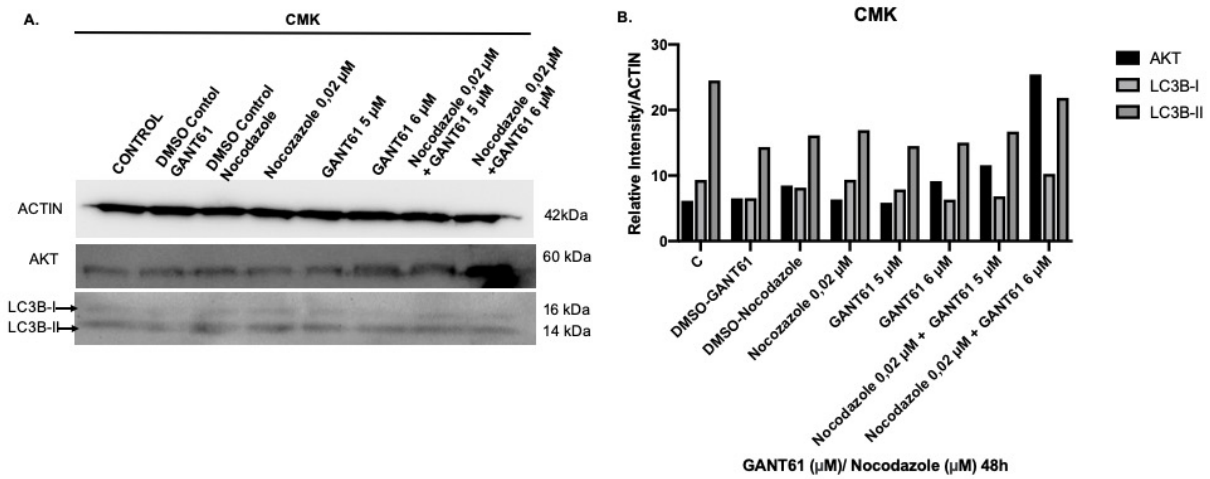


Figure 23. The expression of autophagy markers after autophagy and Hedgehog inhibition for 48hrs in CMK cells.

A. Expression of AKT, LC3I, LC3II and ACTIN (internal loading control) in untreated control, DMSO control (vehicle for GANT61 and Nocodazole), Nocodazole (0.02µM), GANT61 (5 or 6 µM) and two different combinations of GANT61 and Nocodazole. B. The densitometric analysis of AKT/ACTIN, LC3I/ACTIN and LC3II/ACTIN in untreated control, DMSO control (vehicle for GANT61 and Nocodazole), Nocodazole (0.02µM), GANT61 (5 or 6 µM) and two different combinations of GANT61 and Nocodazole.

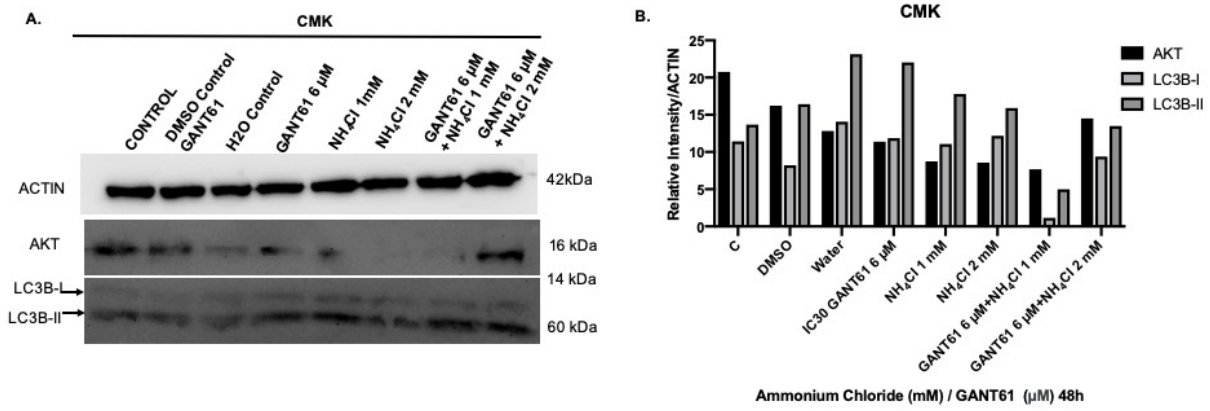


Figure 24. The expression of autophagy markers after autophagy and Hedgehog inhibition for 48hrs in CMK cells.

A. Expression of AKT, LC3I, LC3II and ACTIN (internal loading control) in untreated control, DMSO control (vehicle for GANT61), water control for $NH_4Cl$, $NH_4Cl$ (1 or 2mM), GANT61 (6 µM) and two different combinations of GANT61 and $NH_4Cl$. B. The densitometric analysis of AKT/ACTIN, LC3I/ACTIN and LC3II/ACTIN in in untreated control, DMSO control (vehicle for GANT61), water control for $NH_4Cl$, $NH_4Cl$ (1 or 2mM), GANT61 (6 µM) and two different combinations of GANT61 and $NH_4Cl$.

Our data suggest shows that modulating autophagy is an intricate process that depends on the dose of the treatment, which step of autophagy is inhibited and most importantly the cellular context and the genetic background of the cell lines used. Our results till now are promising where we detected a different response to autophagy and hedgehog inhibition in MOLM-13 compared to CMK cells and more experiments are required to decipher the exact mechanisms that are responsible for that difference. We have tried to check other proteins that are important in the autophagic process such as ULK-1 and ATG5 but the primary antibodies didn't work and we didn't have enough money in our budget to purchase these items.

In majority of cells that were subjected to GANT61 treatment, we detected an increase in the LC3II expression. This suggests an induction of autophagy. Li et al. used a combination of GANT61 and CQ in the context of human hepatic stellate cell line LX-2 cells and similar to our findings, they found that when these cells were treated with CQ and GANT61, the level of LC3II accumulation was higher than single treatment (Li et al., 2017). They deduced that this increase by GANT61 is due to the biosynthesis promotion of LC3II and not the inhibition of LC3II (Li et al., 2017). Other studies have also highlighted the role of GANT61 in increasing the autophagic flux and thus activating autophagy (Wang et al., 2014; Xu et al., 2014; Wang et al., 2018b; Niu et al., 2019). Also, GANT61 treatment was shown to decrease the level of p-AKT and thus activating autophagy in CML (Zeng et al., 2015). Thus, it is essential to check the level of p-AKT in order to understand the mechanisms that led to the activation of the autophagic flux after GANT61 treatment in our cell lines, especially that the level of total AKT is changing depending on the cell line and the treatment.

We have also performed a semiquantitative-PCR for GLI-1, 18-sRNA and GAPDH after treating NB4 and MV4.11 cells with GANT61 (5 and 20 μM). The GLI-1 PCR worked (Figure 25), however we cannot infer anything since the house keeping genes PCR (18-sRNA and GAPDH) didn't work (not shown). In addition to that, the primers that we have ordered for the house keeping gene (18S-RNA and GAPDH) didn't work so we couldn't proceed with our experiments and we had to reorder them (Data not shown).

Despite these problems, we detected a downregulation GLI-1 expression after GANT61 (5 and 20μM) treatment for 48hrs (Figure 25). However, as I mentioned before our house keeping gene primers (GAPDH and 18-sRNA) didn't work (Data not shown). Therefore, we cannot come up with any conclusion.

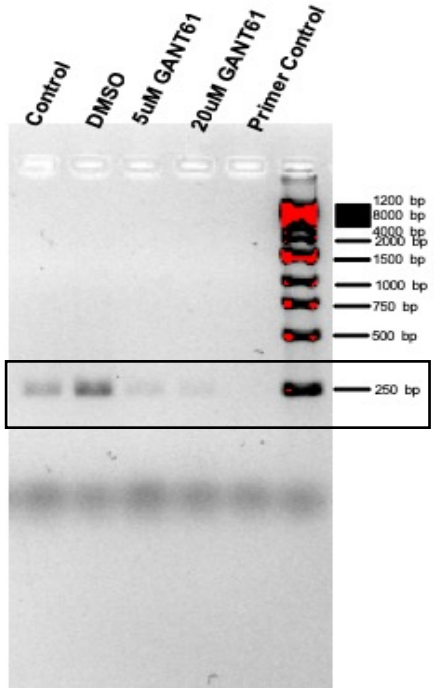


Figure 25. GLI-1 expression decreases after GANT61 treatment for 48hrs. in MV4-11 Cell lines (250bp). GLI-1 band is detected at 250bp.

## 5. Conclusion

Acute myeloid leukemia is a highly heterogenous disease characterized with clonal expansion of immature myeloid progenitors, which leads to a block in differentiation and infiltration into the peripheral blood, bone marrow and other tissue such as the liver and the spleen (Döhner et al., 2015; Ferreira et al., 2015; Morita et al.,2015). Aberrant signaling pathways also plays a role in driving the pathogenesis of AML. Some of the known aberrant pathways that are responsible for AML progression are the Hedgehog, NOTCH, PI3K/AKT/mTOR, WNT, STAT3 and tyrosine kinase pathways (Heidel et al., 2015; Sakamoto et al., 2015; Garg et al., 2019). These pathways crosstalk extensively, thus, promoting the pathogenesis of AML. There has been extensive evidence recently on the fact that AML modulate autophagy so that it acts as a survival and escape mechanism in the context of AML. In this study, we focus on studying the effect of hedgehog signaling pathway on autophagy in the context of the different AML cell lines form different genetic and cytogenetic backgrounds and the effect of this regulation on the leukemogenesis of AML.

We have found that hedgehog inhibition using the specific GLI 1 inhibitor, GANT61, resulted in a significant decrease in the proliferation of all the AML cell lines (NB4, MOLM-13, and MV4-11), however, CMK acute megakaryoblastic leukemia showed a slightly less sensitivity to GLI

inhibition compared to other AML cell lines. Our data shows presents GANT61 as a potent therapy against certain subtypes of AML.

To further understand the effect of this inhibition and based on previous studies that showed HH pathway crosstalks with other pathways among which is the PI3K/AKT/mTOR axes, which is an important regulator of autophagy, we proceeded with checking the effect of autophagy modulation of the survival of the different AML cell lines from different backgrounds before we executed the combination treatment. Our data showed that inhibiting autophagy whether at the autophagosome-lysosome fusion stage (by Vinblastine or Nocodazole) or autophagolysosomal degradation stage (by NH4Cl, CQ or HQ) led to a decrease in the proliferation of AML to a certain degree based on the drug concentration and the timepoint of treatment. These data support data published by Stankov et al. (Stankov et al. 2014). However, the degree of sensitivity varies in the different cell lines at different doses and different time points of autophagy inhibition. Interestingly at low doses of Nocodazole, CMK, which is an AMKL cell line, appear to be more sensitive to autophagy inhibition compared to NB4, MOLM13 and MV4-11 which required higher doses and treatment time. This is due to the fact that they have a higher autophagic flux compared to CMK (Stankov et al., 2014). Moreover, autophagy inhibition led to an increase in the apoptotic cell population in AML cells. On the other hand, Autophagy activation had an effect only on K562 CML cell line but little to no effect on the proliferation of AML cell lines this could be due to the fact that these cells have different basal autophagy levels. We further proceeded to check the effect of inhibition the hedgehog pathway and autophagy on the proliferation of MOLM-13 and CMK cells and we found that MOLM-13 was more sensitive to this combination treatment. This could be due to the fact that GANT61 activates, autophagy, which is not that essential for CMK cells that already have a low level of autophagy (Stankov et al., 2014) and this eliminates the sensitizing effect of autophagy inhibitors. Further experiments are needed to fully understand the role of these pathways. Our preliminary data suggest that GANT 61 in combination with autophagy inhibitor is not a good treatment option for CMK, AMKL cell line, but it is a promising therapy for MOLM-13, *FLT3*- ITD AML cell line.

Moreover, we checked an increase in the level of LC3II due to GANT61 treatment that induces autophagy in our cells. Upon combination treatment of GANT61 with autophagy inhibitors MOLM-13 cell responded by an increase in the level of LC3II. However, CMK increased the level of LC3II only due to GANT61 and nocodazole and not due to GANT61 and $NH_4Cl$ treatment. Also, the level of AKT expression changed depending on the treatment and cell type. These data are promising, however, we have to check the expression level ATG5, ULK-1 p-AKT, p62 and proteins that are involved in autophagy to be able to draw a clear picture of

what is happening in our cells. We have tried ULK-1 and ATG5 but unfortunately our antibodies didn't work.

Thus, suppressing autophagy under a critical threshold allows the abrogation of leukemic growth. This highlights the importance of our study in trying to come up with a combination therapy of autophagy inhibitors in addition to hedgehog signaling pathway inhibitor, in the hope of accomplishing a synergistic effect that may provide a unique approach to target leukemic precursors in this disease.

Till now our data is promising and in accordance with the existing literature, however we had some problems in the delivery of our inhibitors on time, which prevented us from performing more combination treatment experiments on the rest of AML cell lines such as MV4-11 and NB4. As a proof of the delay in delivery, the letter from the company is attached in the financial section of the project. Moreover, the drop in the value of Turkish lira in the past few years made it difficult for us to work and purchase the items within the scope of the budget of the 3001 project. In addition to that, some of the primers and primary antibodies that we have ordered did not work. Our data is promising especially that it will allow the selective targeting of leukemic cells depending on their basal autophagic activity and the activation of the HH pathway. Understanding those context- dependent networks could result in the initiation of the of clinical trials and will open the avenue for new personalized cancer therapies. Moreover, this project is part of the masters thesis and was presented as an oral presentation at a national conference.

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# TÜBİTAK
# PROJE ÖZET BİLGİ FORMU

| | |
|---|---|
| Proje Yürütücüsü: | Dr. Öğr. Üyesi MONA EL KHATIB |
| Proje No: | 216S319 |
| Proje Başlığı: | Akut Myeloid Lösemi Tedavisi için Hedgehog Ve Otofaji Yolaklarının Düzenlenmesi |
| Proje Türü: | 3001 - Başlangıç AR-GE |
| Proje Süresi: | 24 |
| Araştırmacılar: | |
| Danışmanlar: | |
| Projenin Yürütüldüğü Kuruluş ve Adresi: | KAYSERİ ABDULLAH GÜL Ü. YAŞAM VE DOĞA BİLİMLERİ F. |
| Projenin Başlangıç ve Bitiş Tarihleri: | 01/03/2017 - 01/06/2019 |
| Onaylanan Bütçe: | 91438.0 |
| Harcanan Bütçe: | 64289.34 |
| Öz: | Acute myeloid leukemia (AML) is a heterogeneous group of malignancies characterized by clonal disorders with diverse molecular aberrations and dysregulation in signaling pathways. Hedgehog (HH) signaling pathway is an evolutionary conserved signaling pathway that is deregulated in many cancers. HH pathway crosstalks with other pathways among which is the PI3K/AKT/mTOR axes, a main regulator of autophagy, a lysosomal degradation process. These pathways are deregulated in AML. Several studies suggested that autophagy modulation could be an escape mechanism in AML. In this study, we investigated the effect of Hedgehog pathway and autophagy on different subsets of AML cell lines. We have treated CMK, MV4-11, MOLM-13 and NB4 AML cell lines, in addition to K562, CML cells, with GANT 61, GLI1 inhibitor, and different autophagy modulators. MTT assay have showed that GANT61 resulted in a significant decrease in the proliferation of NB4, MOLM-13, and MV4-11, however, CMK showed less sensitivity to GLI inhibition compared to other AML cell lines. Then, we proceeded with checking the effect of autophagy modulation on the survival of the different AML cell lines. Inhibiting autophagy whether at the autophagosome-lysosome fusion stage or autophagolysosomal degradation stage led to a decrease in the survival of AML to a certain degree based on the drug concentration and timepoint of treatment. Combination treatment of autophagy modulators and GANT61 had a synergistic effect in MOLM-13 but not in CMK. GANT61 treatment increased autophagy in AML cell lines that was correlated with an increase in the expression of LC3II detected by western blotting. Also, combination treatment elevated that increase in LC3II in MOLM-13 cell lines but less in CMK cell lines. AKT protein expression changed depending on the type of treatment and cell lines. In conclusion, targeting of Hh and autophagy is a promising therapy against MOLM-13 cell line but not against CMK. |
| Anahtar Kelimeler: | Acute Myeloid Leukemia, Hedgehog, Autophagy, PI3K/AKT/mTOR, Combination Therapy |
| Fikri Ürün Bildirim Formu Sunuldu Mu?: | Evet |
| Projeden Yapılan Yayınlar: | 1- THE FINE TUNING OF THE HEDGEHOG AND AUTOPHAGY PATHWAYS TO COMBAT ACUTE MYELOID LEUKEMIA (Bildiri - Ulusal Bildiri - Sözlü Sunum), |