Merve Şansaçar THE EFFECT OF HEDGEHOG INHIBITION AND AUTOPHAGY MODULATION ON THE PROLIFERATION ANI SURVIVAL OF AML CELL LINES AGU 2019

THE EFFECT OF HEDGEHOG INHIBITION AND AUTOPHAGY MODULATION ON THE PROLIFERATION AND SURVIVAL OF AML CELL LINES

A THESIS
SUBMITTED TO THE DEPARTMENT OF BIOENGINEERING
AND THE GRADUATE SCHOOL OF ENGINEERING AND
SCIENCE OF ABDULLAH GUL UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER

By
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ABSTRACT

THE EFFECT OF HEDGEHOG INHIBITION AND AUTOPHAGY MODULATION ON THE PROLIFERATION AND SURVIVAL OF AML CELL LINES

Merve Şansaçar
MSc. in Bioengineering
Supervisor: Asst. Prof. Mona El Khatib

June, 2019

Acute myeloid leukemia (AML) is a heterogeneous hematopoietic malignancy which occurs as a result of many chromosomal abnormalities such as translocations, deletions or insertions. Aberrant signaling pathways such as PI3K/AKT/mTOR, Notch and Hedgehog pathway have a role in the pathogenesis of AML. Hedgehog pathway (Hh) is a conserved signalling pathway that is important during embryogenesis. It crosstalks with other pathways and regulate autophagy, a cellular degradation and organelle turnover process. Several studies suggested that autophagy modulation could act as an escape mechanism in AML. Given the role of autophagy and Hh in AML, understanding the relationship between autophagy and Hh pathway is important to overcome the leukemic growth. Hence, we checked the effect of Hh inhibition using GANT61 on MOLM-13 and CMK cells using MTT cell viability assay. GANT61 led to a decrease in the both MOLM-13 and CMK cells. After that, we sought to understand the effect of autophagy modulation on CMK and MOLM-13 cell lines and we have found that autophagy inhibitors, NH4CI, Chloroquine(CQ), Hydroxychloroquine and Nocodazole lead to a decrease in the proliferation of CMK and MOLM-13 cell lines. However, PP242, an autophagy activator, had no effect on the proliferation of CMK and MOLM-13 cell lines. Combination treatment of autophagy modulators and GANT61 had a synergistic effect on MOLM-13 but not on CMK. GANT61 treatment increased autophagy in AML cell lines that were correlated with an increase in the expression of LC3B-II detected by western blotting. Also, combination treatment with nocodazole and GANT61 elevated that increase in LC3B-II in both MOLM-13 and 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 pathway, Autophagy

ÖZET

OTOFAJİ MODÜLASYONU VE HEDGEHOG İNHİBİSYONUNUN AML HÜCRE HATLARININ ÇOĞALMASI VE HAYATTA KALMASI ÜZERİNE ETKİSİ

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Akut miyeloid lösemi (AML), translokasyon, delesyon veya insersiyon gibi birçok kromozomal anormallik içeren ve hematopoetik malignite ile sonuçlanan heterojen bir hastalıktır. PI3K / AKT / mTOR, Notch ve Hedgehog yolağı gibi sinyal yollarındaki bozulmalar AML patogenezinde rol oynar. Hedgehog yolağı (Hh) embriyogenez sırasında önemli olan korunmuş bir sinyal yolağıdır. Diğer yolaklarla etkileşime girer ve hücresel bir bozulma ve organel yıkım sürecini oluşturan otofajiyi düzenler. Bazı çalışmalar, otofaji modülasyonunun AML' de bir kaçış mekanizması olarak işlev görebileceğini öne sürmüştür. AML'de otofaji ve Hh rolü göz önüne alındığında, lösemik büyümenin üstesinden gelmek için otofaji ve Hh yolu arasındaki ilişkiyi anlamak önemlidir. Dolayısıyla, GANT61 ile Hh inhibisyonunun AML hücre hatları üzerindeki etkisini MTT hücre canlılığı tahlili kullanarak kontrol ettik. GANT61, AML hücre hatlarında bir azalmaya yol açtı. Bundan sonra, otofaji modülasyonunun AML hücre hatları üzerindeki etkisini anlamaya çalıştık ve otofaji inhibitörleri, NH4CI, Chloroquine (CQ), Hydroxychloroquine ve Nocodazole'ün CMK ve MOLM-13 hücre hatlarının çoğalmasında bir azalmaya yol açtığını gördük. Bununla birlikte, bir otofaji aktivatörü olan PP242, AML hücre hatlarının çoğalmasına etki etmedi. Otofaji modülatörlerinin ve GANT61'in kombinasyon tedavisi, MOLM-13 üzerinde sinerjistik bir etkiye sahipti fakat CMK üzerinde değildi. GANT61 tedavisi, AML hücre hatlarında, western blotlama ile tespit edilen LC3II'nin ekspresyonunda bir artışla ilişkili olan otofajiyi arttırmıştır. Ayrıca, nodadazole ve GANT61 ile kombinasyon tedavisi, hem MOLM-13 hem de CMK hücre hatlarında LC3B-II'de artan bir artış göstermiştir. AKT protein ekspresyonu, tedavi tipine ve hücre hattına bağlı olarak değişti. Sonuç olarak Hh ve otofajinin hedeflenmesi, MOLM-13 hücre hattına karşı umut verici bir tedavidir ancak CMK'ya karşı değildir.

Anahtar kelimeler: Akut miyeloid lösemi, Hedgehog yolağı, Otofaji

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Merve Şansaçar

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Chapter 1

1.Introduction

Acute myeloid leukemia (AML) is a type of acute leukemia and a complex disease, which includes many chromosomal abnormalities such as translocations, deletions or insertions and result in an immature form of myeloid progenitor cells in many organs or tissues [1-4]. According to data of the American Cancer Society, it is estimated that 21,450 new AML cases will be detected in the US in 2019, and approximately 10,920 of these cases are expected to be fatal [5]. Aberrant signaling pathways such as PI3K/AKT/mTOR, Notch and Hedgehog pathways that are essential in stem cell renewal and drug resistance [6] have a role in the pathogenesis of many cancers [7]. One of these pathways is the Hedgehog signaling pathway that is a conserved developmental pathway playing a role in the regulation of many cellular processes such as tissue polarity, cell proliferation and survival during embryogenesis [8-11]. Many studies have indicated that Hedgehog pathways can drive the pathogenesis of AML and can lead to cancer growth [12-16]. The Hh pathway crosstalks with other pathways and has a major role in the regulation of autophagy, which is a cellular degradation and organelle turnover process [17-18]. The role of the autophagic pathway in cancer is controversial. Many studies have shown that depending on the cellular context autophagy can be a pro-cancer or an anticancer mechanism [19-22]. Previous studies have indicated that autophagy has an important role in the progression and pathogenesis of many subtypes of AML [23-24]. Therefore, understanding the link between Hh pathway and autophagy is important to target leukemic growth and treatment of AML. Also, it paves the way to new personalized targeted cancer therapies.

1.1 Acute Myeloid Leukemia

AML is a disease characterized by the transformation of an immature form of myeloid cells in the peripheral blood, bone marrow, spleen and liver that would eventually lead to hematopoietic malignancy [2-4]. Chromosomal aberrations, genetic disorders, and

aberrant signaling pathways could be the drivers of the pathogenesis of AML, which render AML as a highly complex disease [1,12].

1.1.1 Epidemiology

AML is known as the most common form of acute leukemia in adults and its incidence rate is around 80 percent [25]. In Europe, a study has shown that AML is the most common form of myeloid malignancies with the incidence rate of 3.7 per 100.000 year [26]. Although AML is a disease that can be detected at any age, its incidence rate is lower in children compared to adults and it occurs mostly in people aged between 65-74 [27-28]. A study demonstrated that the incidence of AML was 10 times higher in the older population compared to youngsters in Denmark [29]. According to the data of the American Cancer Society, AML is more widespread and fatal in men compared to women. The incidence of new cases is 4.5% in individuals under 20 years of age, however, this rate rises to 25.1% over the age of 65 years. In 2019, 21.450 new AML patients have been estimated to occur in the United States and around 10.920 of these cases are expected to be fatal. In addition to age and gender, the incidence of AML varies according to race. The data presented by the American Cancer Society indicated that AML is more prevalent in whites compared to blacks [5]. Also, in terms of prognosis, Zhao et al. reported that black people have the lowest five-year survival rate compared to white people [30].

1.1.2 Etiology

Several risk factors such as genetic diseases or physical and chemical agents can cause AML or increase predisposition to develop AML [31-32]. AML can develop as a result of many hematopoietic malignancies such as Fanconi's anemia and many genetic disorders such as Down's syndrome [31-32]. For instance, it was reported that a Fanconi's Anemia patient developed AML after allogeneic hematopoietic stem cell transplant [33]. On the other hand, some risk factors that cause the development of AML are external such as, the exposure to a high amount of ionizing radiation or some chemicals such as benzene, which cause chromosomal aberrations leading to AML development [34-35]. Certain environmental risks, such as pesticides or insecticides are related to the etiology of AML. Also, it was revealed that exposure to benzene, diesel fuel, metals or insecticides results in an increased risk of AML [36]. In addition, it was reported that petroleum products, plastics or electrical products may increase the risk of acute leukemia [37].

Although, cigarette smoke and alcohol consumption are not directly acknowledged as the direct risk factors of AML but the consumption of these substances can increase the tendency to develop many cancers including leukemia. Previously, it was reported that smoking increased the risk of AML and they have established a link between the duration of smoking and the increased risk of AML development [38]. Another risk factor for AML is actually the treatment used for cancer such as chemotherapy and radiation therapy. Many studies have shown that chemotherapeutic agents that are used for cancer treatment could increase the tendency to develop AML in the patients receiving them [31-32]. Lois et al reported the development of secondary leukemias in ovarian cancer patients due to chemotherapy treatment [39]. This was also seen in testicular cancer patients that received chemotherapy [40].

1.1.3 Classification of AML

Initially, the classification of AML was performed according to the French-American-British classification system (FAB-M0-M7) (Table 1.1.3.1), which divides AML into different cytochemical and morphological groups depending on cellular differentiation and cytogenetics [41].

Later in 2002, AML was reclassified into different subtypes by the World Health Organization (WHO) based on morphology, cytogenetics and immunophenotyping [42]. It classified AML into six main groups which are AML with recurrent genetic abnormalities, Myelodysplasia-related changes, Therapy-related myeloid neoplasms, AML not otherwise specified (NOS), Myeloid sarcoma and Myeloid proliferations related to Down syndrome. The WHO classification reduced the leukemic blast rate that is required for the diagnosis of AML from 30% (according to FAB classification) to 20% [42]. The WHO classification updated again in 2016 [43]. This update included two main changes in two main categories. The first category is the AML with recurrent genetic abnormalities. In this category they have changed the gene name of MLL to KMT2A. In addition to that, inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2) is not a fusion gene but actually a repositioning of GATA2 enhancer element, which causes the overexpression of MECOM gene and concomitant GATA2 haploinsufficiency [44]. Moreover, the naming of acute promyelocytic leukemia (APL) with t(15;17)(q22;q12); PML-RARA changed to APL with PML-RARA in order to highlight the importance PML-RARA gene fusion. Under the first category, they have also added a new provisional class, which is AML with BCR-ABL1. This addition is important due to the fact that it suggests a specific

treatment for these patients which is tyrosine kinase therapy. Aslo, AML with mutated NPM1 was changed from provisional entities to full entities and AML with CEBPA was limited to biallelic mutations. Moreover, “AML with mutated RUNX1” was added as a new provisional entity. The change in the second category, AML, (NOS) category, included the removal of Erythroleukemia, erythroid/myeloid, which is a subtype of acute erythroid leukemia Table 1.1.3.2) [43].

In addition to the FAB and WHO classification, European LeukemiaNet (ELN) advised a new classification for AML in 2010 [45]. ELN recommended the classification based on prognostic factors such as "Patient-related factors" and "AML-related factors". Under patient related factors, the increase in age is suggested to result in an adverse prognosis. On the other hand, prognostic factors such as cytogenetic and molecular genetic abnormalities or previous chemotherapy agents, which are used for treatment are included in the AML-related factors. AML was classified by ELN into four categories as favorable, Intermediate-I, Intermediate-II and adverse to show a correlation between clinical outcome and genetic abnormalities. Afterwards, these prognostic factors were updated in 2017. According to this update, Intermediate-I and intermediate-II were merged and patients were divided into three prognostic categories. (Table 1.1.3.3) [46].

FAB	Subtype
M0	Undifferentiated acute myeloblastic leukemia
M1	Acute myeloblastic leukemia with minimal maturation
M2	Acute myeloblastic leukemia with maturation
M3	Acute promyelocytic leukemia (APL)
M4	Acute myelomonocytic leukemia
M5	Acute monocytic leukemia
M6	Acute erythroid leukemia
M7	Acute megakaryoblastic leukemia

Tablo 1.1.3.1 French-American-British classification system for AML [41]

Acute myeloid leukemia (AML) and related neoplasms
AML with recurrent genetic abnormalities
t(8;21)(q22;q22.1);RUNX1-RUNX1T1
inv(16)(p13.1q22) or t(16;16)(p13.1;q22);CBFB-MYH11
APL with PML-RARA
t(9;11)(p21.3;q23.3);MLLT3-KMT2A
t(6;9)(p23;q34.1);DEK-NUP214
inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2, MECOM
AML (megakaryoblastic) with t(1;22)(p13.3;q13.3);RBM15-MKL1
Provisional entity: AML with BCR-ABL1
NPM1 gene mutations
Biallelic mutations of *CEBPA*
Provisional entity: AML with mutated RUNX1
Myelodysplasia-related changes
Therapy-related myeloid neoplasms
AML, NOS
AML with minimal differentiation
AML without maturation
AML with maturation
Acute myelomonocytic leukemia
Acute monoblastic/monocytic leukemia
Pure erythroid leukemia
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferations related to Down syndrome
Transient abnormal myelopoiesis (TAM)
Myeloid leukemia associated with Down syndrome

Table 1.1.3.2 The World Health Organization (WHO) classification of AML in 2016 [43]

Risk Category	Genetic abnormality
Favorable	t(8;21)(q22;q22.1); RUNX1-RUNX1T1
	inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11
	Mutated NPM1 without FLT3-ITD or with FLT3-ITD^{low}
	Biallelic mutated CEBPA
Intermediate	Mutated NPM1 and FLT3-ITD^{high}
	Wild-type NPM1 without FLT3-ITD or with FLT3-ITD^{low} (without adverse-risk genetic lesions)
	t(9;11)(p21.3;q23.3); MLLT3-KMT2A
	Cytogenetic abnormalities not classified as favorable or adverse
Adverse	t(6;9)(p23;q34.1); DEK-NUP214
	t(v;11q23.3); KMT2A rearranged
	t(9;22)(q34.1;q11.2); BCR-ABL1
	inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2,MECOM(EVI1)
	−5 or del(5q); −7; −17/abn(17p)
	Complex karyotype, monosomal karyotype
	Wild-type NPM1 and FLT3-ITD^{high}
	Mutated RUNX1
	Mutated ASXL1

Table 1.1.3.3 Prognostic Factors based on the European LeukemiaNet (ELN) in 2017 [46]

1.1.4 Diagnosis of AML

AML is diagnosed based on the morphological, cytogenetic, molecular and immunophenotypic properties of AML blasts. Morphologically, the recommendation according to the WHO for the diagnosis of AML is the presence of 500 nucleated cells in the bone marrow smears and the myeloblasts should be ≥ 20%. Furthermore, regardless of the blast number, the detection of cytogenetic abnormalities is considered sufficient for AML diagnosis [43].

Another method of diagnosis is immunophenotyping, which is based on the cluster of differentiation on the surface of the AML cells [47]. The detection of the expression of the following cell surface markers CD13, CD14, CD33, CD34, CD56, and TdT (the terminal deoxyribonucleotidyl transferase) is an indication of poor prognosis in AML cells [48-51]. CD13 and CD33, which are known myeloid-lineage associated cell surface markers, the two myeloid-lineage-associated markers, in the presence of MPO and the lack of lymphoid markers are used in the detection and identification of AML [52-53]. Moreover, HLA-DR, a class II MHC, is often used for the identification of AML except promyelocytic M3 AML (APL), however it is not specific since it is also present on acute undifferentiated leukemias, Acute B Lymphoblastic Leukemia, normal B cells,

monocytes and activated T-cells [54]. Moreover, CD117, the immature myeloid marker, is associated with AML halted during the early steps of differentiation [55]. Also, myeloperoxidase (MPO) is an enzyme that appears during myeloid differentiation and is an indicator of myeloid lineage [56]. All together, the expression of cell-surface and cytoplasmic markers in addition to enzymes allows us to identify the different maturation and diffierentiation levels of AML [47].

Cytogenetic analysis is one of the main diagnostic methods that is used in the clinic for the detection of AML. Recently, the new sequencing technologies allowed to discover many genetic aberrations in AML. Another method that is feasible nowadays due to the increase in the senstitivity of reverse transcriptase–polymerase chain reaction (RT-PCR) is molecular testing that is able to detect the presence of driver mutations that lead to AML such as, Nucleophosmin 1 (NPM1), CCAAT Enhancer Binding Protein α (CEBPA), Runt-Related Transcription Factor (RUNX1), or FMS-like tyrosine kinase (FLT3) gene mutations lead to AML [46].

1.1.5 Current Treatment for AML

AML is a complex disease comprised of different chromosomal abnormalities and aberrant signaling pathways, therefore conventional treatment methods are not very successful in combatting AML and this suggests the need for novel therapies against AML. [1, 6]. The most common treatment methods in the clinic that are currently used for the treatment of AML are chemotherapy, immunotherapy and stem cell transplantation.

The chemotherapy regimen for AML is generally divided into two sections: Induction and Consolidation. The aim of induction therapy is reducing and clearing the number of leukemic blasts. Drugs that are commonly used in induction are cytarabine, anthracycline and cladribine. Usually, induction therapy includes intensive anthracycline and cytarabine regimen "7 + 3" [57-58]. The second step of the chemotherapy regimen is consolidation therapy, which involves intensive chemotherapy to prevent the relapse of the treated AML patients and usually utilizes very high doses of cytarabine. [59]. However, chemotherapy has a lot of side effects, which lead to pain, infection, bleeding and fatigue [59, 60]. Due to these side effects, new targeted therapy approaches are investigated for the successful treatment of AML. Many genetic abnormalities and

aberrations of signaling pathways are known to be involved in the pathogenesis of AML [1, 11]. Targeting directly these mutations or aberrant pathways with specific inhibitors against the single mutations or pathway or multiple targeted therapies against more than one mutation presents a new window for the treatment of AML. Hence, the wealth of information coming out of the whole genome and exome sequencing fueled the research in the field of targeted therapy in AML. For instance, the first promising targeted therapy, all-trans retinoic acid (ATRA), is a successful and promising targeted therapy used to treat acute promyelocytic leukemia (APL) patients [61, 62]. These patients carry a PML-RARA gene fusion [63]. The success of ATRA in the clinic paved the way for more studies. Midostaurin and Gilteritinib, multikinase inhibitors, were approved by the Food and Drug Administration (FDA) for the AML treatment that harbor aberrations in the Fms-Like Tyrosine Kinase (FLT3) [64-67]. In addition to that, sorafenib, a tyrosine kinase inhibitor, that was already approved by FDA for many other types of cancer is now under in clinical trial for AML patients harboring FLT3 mutations [68]. Recently, the FDA has approved other targeted therapies for AML such as Venclexta, which targets BCL-2 protein, and glasdegib, Hh pathway (Table 1.1.5.1) [69, 70].

Another alternative method for the treatment of AML is immunotherapy and it involves antibody-based therapy and adoptive T cell therapy [71]. In the context of antibody-based therapy, the myeloid cell surface marker, CD33, is the most suitable candidate for immunotherapy since it is expressed by approximately 80% of AML cells. Gemtuzumab ozogamacin (GO) is the first anti-CD33 approved by the FDA [72]. CD33 drugs are also classified under targeted therapy in AML. However, in 2010, GO was withdrawn from the market by the FDA due to its toxic level and the negative results of the clinical outcomes during phase III study [73-75]. In addition to that, clinical studies are currently ongoing for other monoclonal antibodies against CD33 such as vadastuximab talirine and AMG 330 [68]. Other cell surface markers have been suggested as alternative successful treatment options for AML. Krupka et al. have shown that an Fc-engineered antibody targeting CD157, MEN 1112, can be used as a promising therapy against primary AML cell lines [76].

The other method in immunotherapy is the usage of adoptive T-cells to treat leukemia, which includes the application of a specific type of T cell on AML patients [77]. T cells that are genetically modified to express cell membrane-bound receptors also called chimeric antigen receptors (CARs) T cells are the most studied treatment option

for different hematopoietic malignancies such as acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), lymphoma or multiple myeloma [77-78]. This treatment method involves the genetic engineering of the patient's autologous T cells to express CARs against specific antigens and the transplantation of these cells back to the patient. CD33 is the best candidate antigen for CAR T cells because it is expressed in the majority of AML patients [79]. A study has revealed the anti-leukemic effect of CAR-33 T cells in the human AML cells and humanized mouse models [80]. Another cell surface marker that is overexpressed in AML is CD123, an alpha-chain of the interleukin-3 receptor, is another alternative target for CAR T cell therapy [81-82]. Moreover, the anti-leukemic activity of CAR123 T cells in the xenogeneic model of AML was reported [83]. In addition, Pizzitola et al. demonstrated that genetically modified CAR123 T cells decreased leukemic burden in vivo without affecting hematopoiesis [84]. In March 2017, FDA approved UCART123, which is a CAR T-cell therapy targeting CD123 and phase I clinical trials were initiated for AML patients [85]. However, in September 2017, FDA lifted 2 phase I clinical trials for UCART123 since it resulted in the death of AML patients due to Cytokine Release Syndrome (CRS) after 9 days of receiving the treatment [86]. On 7 November 2017, trials re-started with the application of different provisions such as reduction of the dosage of CART-123, age limitation to patients under 65 years and no organ dysfunction at the time of the treatment [87]. However, majority of the targeted cell surface markers are also found on normal myeloid cells, thus, there is no AML-specific antigen that CAR T cells can specifically targets without damaging normal hematopoiesis. Therefore, CAR T cell therapy faces some challenges. For instance, O'Hear et al. used CART33 cells to decrease the tumor burden in NSG mice, however, this treatment caused significant on-target off-leukemia toxicity and eradication of normal myeloid cells and HPCs [88].

Another successful method for the treatment of AML is stem cell transplantation. Stem cells are undifferentiated cells with the ability of self-renewal and differentiation to several types of cells in the body. Due to the fact that stem cells are involved in tissue repair and regeneration, researchers are interested in stem cell transplantation for the treatment of many cancer [89]. In 1957, the researchers reported the first successful bone marrow transplant and the number of AML patients receiving this treatment has continuously increased since then due to its success [90]. Hematopoietic stem cell transplantation (HSCT) is used as post-consolidation treatment in the AML patients [91].

However, there are some side effects to allogeneic SCTsuch as graft-versus-host disease (GVHD) [92]. Hence, AML is a complex disease and the thorough understanding of the molecular aberrations and mutations that drives the leukemogenesis process of AML is essential for the design of more targeted and personalized therapies against it.

Drug	Classification	Target
Trisenox (Arsenic Trioxide)	Antineoplastic" or "cytotoxic"	Acute promyelocytic leukemia (APL)
Cerubidine (Daunorubicin Hydrochloride)	Anthracycline antitumor antibiotic	With other drugs as remission induction therapy
Cyclophosphamide	Alkylating agent	Alone or with other drugs
Cytosar-U (Cytarabine)	Nucleic Acid Synthesis Inhibitor	With other drugs
Vyxeos (Daunorubicin Hydrochloride and Cytarabine Liposome)	antineoplastic" or "cytotoxic"	Patients with therapy-related AML (tAML) or AML with myelodysplasia-related changes (AML-MRC).
Daurismo (Glasdegib Maleate)	Hh pathway inhibitor	Smoothened (SMO)
Idamycin PFS (Idarubicin Hydrochloride)	Anthracyline antitumor antibiotic	With other drugs
Idhifa (Enasidenib Mesylate)	IDH2 inhibitor	IDH2
Mitoxantrone Hydrochloride	Antitumor antibiotic	With other drugs
Mylotarg (Gemtuzumab Ozogamicin)	Antibody-drug conjugate consisting of a monoclonal antibody	CD33
Rydapt (Midostaurin)	FLT3 inhibitor	FLT3
Tabloid (Thioguanine)	Antimetabolite	Remission induction therapy and remission consolidation therapy
Tibsovo (Ivosidenib)	IDH1 inhibitor	IDH1
Venclexta (Venetoclax)	Inhibitor of BCL-2 protein	Combination with azacitidine, decitabine, or low-dose cytarabine
Vincristine Sulfate	Antimitotic and antineoplastic (plant alkaloid)	Acute leukemia
Xospata (Gilteritinib Fumarate)	FLT3 inhibitor	FLT3
Drug Combinations Used in Acute Myeloid Leukemia (AML)		
ADE	A= Cytarabine (Ara-C) D= Daunorubicin Hydrochloride E= Etoposide Phosphate	Acute myeloid leukemia in children

Table 1.1.5.1 Drugs Approved for AML by FDA [93]

1.1.6 Molecular abnormalities in AML

1.1.6.1 Genetic aberrations

Recently, due to the extensive use of whole genome and exon sequencing, many genetic aberrations were discovered in AML such as KIT, FLT3, RAS, DNA Methyltansferase 3A (DNMT) or Isocitrate Dehydrogenase (IDH). [1]. To highlight the importance of these aberrations the WHO added a group, "AML with recurrent genetic abnormalities", to characterize and classify AML harboring mutation in *RUNX1, NPM1 CEBPA* genes [43]. Molecular analysis of these genetic aberrations allows the diagnosis of AML and facilitates the treatment of AML.

FLT3 Mutations

FLT3 is a type of receptor tyrosine kinase and it important in hematopoiesis. Hematopoietic stem or progenitor cells normally express FLT3 and it is important for HSPCs lineage development. It is also involved in stem cell differentiation and proliferation [94-96]. It is mutated in AML patients and the two main mutations are Internal tandem duplication (ITD) in the juxtamembrane domain and point mutation of the tyrosine kinase domain (TKD) [97]. FLT3 ITD occurs in 23 % of AML cases and it is located in exons 14-15 [98]. It is usually an indication of poor prognosis in AML patients and it is the most common FLT3 mutation compared to FLT3 TKD [99-100]. Point mutations in the codon 835 within the FLT3 TK domain affect the activity of tyrosine kinase and lead to the consititutive activation of tyrosine kinase, thus triggering the overgrowth of AML cells. FLT3 TKD mutations are detected in 7% of adult AML patients [101-103]. Standard consolidation chemotherapy is used to treat patients with FLT3 mutation. Also, a targeted therapy directed against FLT3 mutations is currently used in the clinic. Recently, two tyrosine kinase inhibitors, Midostaurin and Gliteritinib were approved by the FDA for treatment of FLT3 mutated AML in 2017 [64-67].

DNMT3A Mutations

Mutations in proteins involved DNA methylation has been linked to the development and progression of many types of cancers and in hematopoietic malignancy such as AML [104]. DNA methyltransferase 3A (DNMT3A) catalyses the methylation of cytosine into 5-methylcytosine [105]. DNMT3A mutations occurs in % 20 of AML patients [106]. The substitution of the amino acid arginine at codon 882 (R882) is the

most common mutation in DNMT3A [107]. Mutations in DNMT3A often co-occur with *ASXL1, FLT3, IDH1-2,* and *TET2* genes [108]. It has been reported that the mutation in DNMT3A causes poor prognosis in AML [109, 110]. A study has revealed that inhibition of DOT1L (disrupter of telomere silencing 1-like) which is an important player in the leukemogenesis process of primary AML samples with DNMT3A mutations. This study has shown that DOT1L inhibitor, EPZ5676, has an anti-leukemic potential for DNMT3A-mutated AML [111, 112]. In addition, the hypomethylating agent decitabine, which is also a known DNMT inhibitor is currently in clinical trials as a promising treatment to prevent leukemic growth [113].

NPM1 Mutations

The *NPM1* gene is located on chromosome 5 (5q35) and it is related to genomic stability, cell cycle progression, and apoptosis [114]. It acts as a chaperone protein that facilitates the transport of ribosomal proteins from the nucleus [115]. In addition to that, NPM1 has a significant role in the regulation of apoptosis. In the nucleolus, p14ARF, tumor suppressor gene, and NPM1 form a dimer. Under stress, NPM1 and p14ARF dissociate and bind to MDM2, which is a negative regulator of p53. This prevents the proteasomal degradation of p53 via MDM2, thus allowing p53 to induce the transcription of different genes that are related to apoptosis. Therefore, the mutation in NPM1 promotes AML cell survival via regulation of apoptosis. [116]. *NPM1* gene mutations occur in exon 12 and they are common mutation in AML (35%-60% of all AML patients). It leads to an abnormal NPM1 protein expression in the cytoplasm instead of the nucleus and these mutations are associated with promotion of leukemogenesis [117-118]. NPM1 gene mutations are generally co-founded with DNMT3A and FLT3-ITD mutations [119]. Interestingly, NMP1 has been shıuld to have a particular genetic signature where this mutation was found with a downregulated expression of CD34 and upregulated expression of the *HOX* gene [120-121]. The prevalent treatment for AML-mutated-NPM1 patient is induction and remission chemotherapy and it usually gives a good response [122].

IDH Mutations

The *IDH1* and *IDH2* genes, which are located on chromosome 2q33 and 16q26, respectively, encode IDH1 and IDH2 catalytic enzymes responsible for histone demethylation and DNA modification [123]. IDH1 and IDH2 catalyze the oxidative

decarboxylation of isocitrate to form a-ketoglutarate (α-KG). However, mutations at Arg^{132} of IDH1 and Arg^{172} of IDH2 results in a new enzymatic activity that cause the catalytic reduction of α-KG into 2-hydroxyglutarate that results in alterations in epigenetics and a block in differentiation that results in pathogenesis [124]. Mutations in the IDH affect the many different cellular processes including cell proliferation and differentiation [125]. While IDH1 mutations occur in 6–16% of AML cases, mutations in IDH2 are seen in 8–19% [126]. IDH-specific inhibitors such as Ivosidenib and Enasidenib are novel therapies that are used in the clinic for the treatment of IDH-mutated AML [127].

RUNX1 Mutations

RUNX1, which is a transcription factor also known as acute myeloid leukemia 1 protein (AML1), is widely expresses by hematopoietic cells and essential for hematopoietic differentiation [128]. Especially, the t(8;21) translocation, which involves the AML1 and ETO genes, and and leads to the expression of AML1-ETO fusion protein occuring about 10% of AML cases [129]. RUNX1 mutation is usually an indication of poor prognosis in AML cases [130-132].

TET2 Mutations

TET2 (tet methylcytosine dioxygenase 2) resides at chromosome 4q24 and since TET2 catalyzes the conversion of 5-methylcytosine to 5-hydroxymethylcytosine, thus it is involved in epigenetic regulations [133, 134]. TET2 mutations were detected in 2009 for the first time by single nucleotide polymorphism (SNP) in hematopoietic malignancies [135]. These mutations are associated with abnormal hematopoiesis [136]. Mutations in the *TET2* gene result in a decrease in the level of hydroxymethyl cytosine by increasing DNA methylation. These mutations were detected in 9%–23% of AML patiens. [137-138]. The incidence of TET2 mutations is higher in elderly patients compared to young patients [139, 140]. They are usually correlated with poor prognosis in AML patients [141-142]. TET2 mutation generally coexists with NPM1 and uncommonly with mutations in IDH1 and IDH2 [143-144].

CEBPA Mutations

CEBPA is a transcription factor found on the 19q13.1 chromosome and it is associated with neutrophil differentiation [145]. CEBPA mutations block the differentiation of myeloid lineage and this results in leukemia. Patients with CEBPA mutations usually carry two mutations ($CEBPA^{double\text{-}mut}$) that is usually comprised of an N-terminal frameshift mutation and a C-terminal insertion mutation [146]. CEBPA mutations are detected in nearly 5% -14% of AML cases [147]. In AML patients with normal karyotype, biallelic mutations are usually related to a good prognosis and low risk of relapse and it positively affect the survival of these patients. [148-149]. Recently, in 2016, WHO has identified biallelic mutations of CEBPA as full entities and these mutations were classified under favorable prognosis according to the ELN classification [43], [46].

ASXL1 Mutations

ASXL1 gene is localized on chromosome 20q11 [150-151]. Gelsi-Boyer et al. were the first research group to detect mutated-ASXL1 in myelodysplastic syndromes. [152]. ASXL1 mutation is known as the second most frequent mutation in hematopoietic malignancies [153]. Different studies indicated that ASXL1 mutation was detected in about 6 to 30% of AML [154-156]. In addition to that, it is more prevalent in patients older than 60 years old, and is rare in children [157].

MLL Mutations

MLL gene is located on chromosome 11q23 [104]. It has a role in embryogenesis and hematopoiesis. The abnormal activation of the *MLL* gene is related to leukemia and the increase of leukemic cell proliferation [158-159]. Translocations in the *MLL* gene lead to AML. The most common MLL translocation in AML is t (9; 11) (p22; q23) translocation with a frequency of approximately 2% and according to ELN classification, it falls under the intermediate prognostic risk group [160]. In addition, MLL-ENL and MLL-FKBP fusion proteins have been shown to cause HOX gene overexpression which leads to poor prognosis in AML [161]. EPZ-5676, which is a known DOT1L inhibitor, offers a specific therapy window for MLL by preventing leukemic growth. This is due to the fact that DOT1L is necessary for the development of MLL-rearranged leukemia and the inhibition of DOTL1 prevents leukemic growth [162].

TP53 Mutations

TP53 is a tumor suppressor and has a role in different cellular processes including apoptosis and DNA repair [104]. Mutations in *p53*, which is located on 17p13.1, lead to uncontrolled proliferation of cells and eventually cancer development [163]. Mutations in this gene account for about 15% of treatment-related AML and 70-80% of patients with a complex karyotype. Mutations in TP53 leads to poor survival [119-164]. As a therapeutic approach to TP53-mutated AML, PANDA, a long non-coding RNA, has been shown to stabilize the mutant TP53, thus restoring its anti-cancerous activity [165].

KIT Mutations

KIT is a receptor tyrosine kinase and *KIT* gene is found on chromosome 4q12 [1, 166]. Mutations in *KIT* are related to poor prognosis and are shown in 2%–14% of AML patients [167]. KIT, which is stem cell factor, has a role in several regulatory pathways such as JAK/STAT, PI3K or MAPK signaling pathways and mutation in this factor result in constitutive activation of these pathways [168]. Patients with KIT mutations tend to relapse after treatment [169]. Multikinase inhibitors such as midostaurin, dasatinib, imatinib or sunitinib are used to treat mutated KIT [170]. It was reported that APcK110, which is a KIT inhibitor increased the survival of the xenograft mouse model via inhibition of proliferation of the AML cells [171].

RAS Mutations

The *RAS* genes which are encoded a family of membrane-associated proteins play a role regulation of signal transduction. Three functional RAS genes have been identified consisting of N- (from a neuroblastoma cell line), K- (Kirsten) and H- (Harvey) RAS genes. Mutation in RAS gene was reported to be associated with poor outcome in AML [172]. RAS mutations occur approximately 10% to 15% of AML cases [104]. Point mutations in *RAS* increase the RAS-GTP levels by reducing the susceptibility of RAS proteins to guanine triphosphate (GTP) activating protein [174].

1.1.6.2 Aberrant Signaling Pathways

In addition to genetic abnormalities, aberrant signaling pathways such as the hedgehog, Notch, PI3K/AKT/mTOR, Wnt, STAT3 and tyrosine kinase pathways drive the pathogenesis of AML [6]. Self-renewal is crucial and necessary for both

hematopoietic stem cells (HSC) and leukemic stem cells (LSC). Some evolutionarily conserved signaling pathways are related to stem cell renewal and resistance to chemotherapeutic agents. [6, 7]. Notch signaling has a role in the development, stem cell self-renewal and differentiation in hematopoiesis and, dysregulated Notch signaling have been detected in many hematological malignancies [5, 175]. Wnt is another signaling pathway regulating development and hematopoiesis. Aberrant Wnt signaling pathway has a role in the initiation and development of hematologic malignancy because Wnt in pathways involved in proliferation and self-renewal [6,176]. Another important pathway that drives the pathogenesis of AML is PI3K/AKT/mTOR signaling axis, which is composed of Phosphatidylinositide 3 kinase (PI3K) and its downstream mediators AKT and mammalian target of rapamycin (mTOR). This pathway regulates many cellular processes including cell differentiation, cell proliferation, survival and metabolism [177]. The PI3K/AKT pathway is constitutively activated in AML and activation of the PI3K/mTOR/AKT pathway has been shown in %95 percent of primary AML samples [178]. The hedgehog (Hh) pathway has a role in embryogenesis and normal adult homeostasis, however, aberrant Hh pathway could drive cancer development [8]. Recent evidence has demonstrated that hedgehog and the PI3K/AKT/mTOR pathway interplay and together lead to the pathogenesis of AML [179-181]. PI3K/AKT/mTOR axis has a role in the inactivation of Gli, through protein phosphatase 2A (PP2A) which acts as an antagonist to the mTOR complex [182].

1.2 The Hedgehog (Hh) pathway

The Hedgehog pathway is an evolutionary conserved signaling pathway, which is related to variable cellular processes including proliferation, tissue polarity and survival, in addition it has an essential role in the embryonic development [8-10], [183]. The Hh pathway is usually inactivated in adults and reactivated when necessary to regulate cellular homeostasis [9]. The mechanism of the Hh pathway has first been revealed in 1980s. They discovered a set of genes in the *Drosophila* and found that mutations in these genes correlated with the disruption of normal body segmentation of the larvae. One of those genes was the hedgehog gene and the name as 'hedgehog' was derived from this spiny phenotype in the D. melanogaster larvae. The mutant Hh gene led to mutant larvae covered with denticles similar to the spines of hedgehog [184].

Hh signaling pathway includes various protein families such as Hh ligands (SHH, IHH, DHH), the twelve-transmembrane domain receptor protein Patched (Ptch1/2), which is located in the primary cilium, the seven-transmembrane G-protein associated receptor Smoothened (Smo), which transmits the Hh signal, the suppressor of fused protein (SuFu) and the glioma-associated oncogene (Gli) proteins (Gli1, Gli2 and Gli3), which are known transcription factors [185].

Three Hh family members were identified in mammals: Sonic hedgehog (SHH), Indian hedgehog (IHH), and Desert hedgehog (DHH) [7]. These names were inspired by the Sega computer game character, a kind of hedgehog in Egypt and an endemic hedgehog in Pakistan, respectively [186]. SHH is the most-studied among the Hh proteins and it has a role in the development of most organs, tissues and structures such as front part of the brain, tooth or eyes during the early embryonic stages [8]. Defects in the SHH ligand leads to holoprosencephaly, which is a disease characterized by incomplete separation of the two hemispheres [187-188]. Other Hh ligands are less studied compared to SHH. DHH is expressed in the gonads and it is essential for differentiation of germ cells and testis development, while IHH ligand has a role in bone formation [189-190]. Hh proteins undergo some post-transcriptional modifications: Hh protein is synthesized as a 45 kDa precursor protein with the N-terminal signaling domain (N-Hh) (19kDa) and C terminal processing domain (C-Hh) (26 kDa). It is autoproteolytically cleaved and the N-terminal domain is removed from the precursor protein. Modifications are followed by the addition of cholesterol and palmitic acid to the N-Hh domain. Modified Hh protein is secreted to the exterior of the cell membrane by Dispatched (Disp) protein and the start of signal transduction is initiated upon the binding of the ligand to the receptor. [191-193].

When Hh ligand is absent, the activation of Smo protein in the primary cilium is suppressed by the Ptch receptor and Smo cannot enter the plasma membrane. Therefore, Gli proteins remain with suppressor of fused (SuFu) protein complexes, which has a role in preventing the translocation of Gli proteins into the nucleus [183]. Protein kinase A (PKA), glycogen synthase kinase-3 (GSK3), and casein kinase 1 (CK1) lead to the conversion of Gli activator (GliA) to Gli repressor (GliR) by phosphorylating Gli proteins [194-196]. The full-length Gli proteins are converted to the truncated form, which is GliR. The truncated form of Gli, which is formed by the processing of proteasome, translocates

into the nucleus and this condition causes the inactivation of Hh target genes [183]. On the other hand, in the presence of Hh ligands, Ptch is inactivated by Hh protein. In addition, some proteins such as CAM-related/down-regulated by oncogenes (CDO), Brother of CDO (BOC), Growth arrest-specific 1(GAS1) and LDL receptor-related protein 2 (LRP2) promotes the binding of Hh ligand to PTCH [197-199]. The Hh-Ptch complex leaves the membrane and it is degraded by the proteasome. Thus, the inhibitory effect of Ptch on Smo is removed [183]. Smo translocates into the primary cilium which is essential for the activation of Gli. When SMO is on the primary cilium, the kinesin protein Kif7 mediates the movement of the Gli protein to the primary cilium [200]. The Gli proteins separate from the SuFu-Gli complex and phosphorylation of Gli proteins by protein kinases is prevented. Thus, Gli protein is not degraded by the proteasome. Gli proteins maintain the full-length form and they translocate and enter the nucleus. This results in the activation and the expression of Hh pathway target genes (Figure 1.2.1) [183].

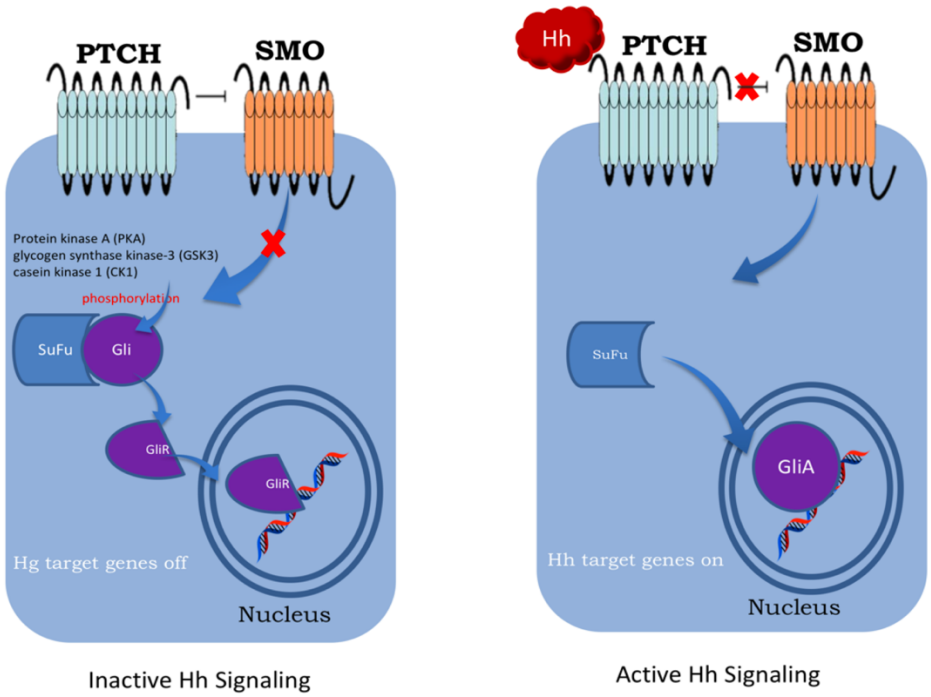

Figure 1.2.1 Schematic illustration of the Hedgehog signaling pathway. (Adapted from Hedgehog Signaling in Fibrosis Liping Hu, Xiangyang Lin, Hong Lu, Bicheng Chen and Yongheng Bai Molecular Pharmacology February 1, 87, 174-182 (2015)).

1.2.1 Aberrant Hh pathway in AML

Recent studies related to the pathogenesis of AML have indicated that the Hh pathway has a crucial role in the regulation of the self-renewal capacity of hematopoietic stem cells [12]. As mentioned before, Hh pathway is associated with many cellular processes, from cell differentiation to tissue polarity [11]. Although the Hh pathway is generally inactive in healthy adults, the activation of Hh pathway is detected in the pathogenesis of many hematological malignancies and solid tumor such as glioblastoma, medulloblastoma, cholangiocarcinoma, pancreatic cancer, basal cell carcinoma [201-206]. Several studies have shown that inhibition of the Hedgehog pathway can decrease the chemoresistance and may overcome leukemic growth. Sadarangani et al. reported that inhibition of GLI transcription factor abolished the leukemic progression [207]. In another study, a combination of SMO and internal tandem duplications (ITD) of FLT3 inhibitors decreased the tumor growth in FLT3-mutated AML [15]. Moreover, Kobune et al. revealed that inhibition of Hh signaling pathway reduced drug resistance in CD34(+) leukemic cells [16]. In addition, targeting GLI1 with Hh inhibitor overcame drug resistance by enhancing sensitivity to cytarabine and prevented AML CD34+ cell growth and this case again revealed the therapeutic effect of targeting Hh pathway in AML [16]. Further evidence supporting Hh signaling pathway inhibitors as an anti-leukemic agent was revealed by Zou et al. who proved the presence of a correlation between GLI1 expression and DNMT1 in MDS. The silencing of the Gli gene in MDS increased the effect of 5-aza-2'-deoxycytidine and decreased the expression of DNMT1 in MDS cells. Thus, combination of Gli1 inhibitors and demethylating agents could combat MDS [208]. It has been shown that the combination of SMO inhibitor with chemotherapeutic drugs decreased the chemoresistance and prevented AML progression [209]. Also, it has been shown that the Hedgehog pathway crosstalk with pathways regulating autophagy and the aberration in these pathways could drive the pathogenesis of many cancers.

1.3 Autophagy

Autophagy is a catabolic process essential for the degradation of cellular components, such as impaired organelles and unnecessary proteins or lipids and it has a role in cellular homeostasis. [17-18, 210]. Christian de Duve defined autophagy as a self-eating mechanism in 1963 [211].

1.3.1 Molecular mechanism of autophagy

Autophagy is initiated with the formation of autophagosomes at the phagophore assembly sites (PAS) [212]. Autophagy is usually activated in response to starvation. In addition to that, some chemicals such as rapamycin or PP242 can cause the activation of autophagy. Autophagy can also be triggered due to hypoxic condition, oxidative stress or ER stress [212].

More than 30 'Autophagy-related' (ATG) proteins, which were firstly identified in yeast, are involved in the regulation of the autophagic process such us formation of double-membrane vesicles, fusion to lysosome and degradation [213]. The first step of autophagy is initiated by the Unc-51-like kinase (ULK) complex, which is composed of ULK1/2 (Ser/Thr protein kinase ULK1), RB1-inducible coiled-coil protein 1 (FIP200), autophagy-related protein 13 (ATG13) and Autophagy-related protein 101 (ATG10) [213]. The ULK complex is necessary for the initiation of the double-membrane vesicle, called autophagosome [214]. Vesicular sorting protein 34 complex/phosphatidylinositol-3-phosphate kinase III (Vps34/PI3PIII) is composed of Beclin1, VPS15, and ATG14 and are required for the proper phagophore formation [214]. ATG5-ATG12 and the microtubule-associated protein 1 light chain 3 (LC3) system are two ubiquitin-like conjugation systems and regulate the elongation and closure of the autophagosome. Firstly, ATG7 (like an E1 ubiquitin-activating enzyme) and ATG10 (an E2 ubiquitin-conjugating enzyme) conjugate ATG12 to ATG5. This ATG12–ATG5 conjugate binds ATG16L to form the multimeric ATG12–ATG5–ATG16L complex [215-216]. In the second ubiquitin-like system, LC3 (Microtubule-associated protein 1 light chain 3) conjugates to the lipid phosphatidylethanolamine (PE) and this process results in the lipidation of cytosolic soluble LC3-I to LC3-II, which is associated to the autophagosome [217-218]. The process of autophagosome maturation and fusion with lysosomes is regulated by GTPase RAB7A (member RAS oncogene family), the homotypic fusion and protein sorting (HOPS) complex, SNAREs, soluble N-ethylmaleimide-sensitive factor activating protein receptor proteins, which is included syntaxin 17 (STX17) and synaptosomal-associated protein 29 (SNAP29), and vesicle- associated membrane protein 8 (VAMP8). In the final step, autophagosomes fuse with the lysosomes that contains degradative enzymes, and lead to the formation of autophagolysosome (Figure 1.3.1.1) [213].

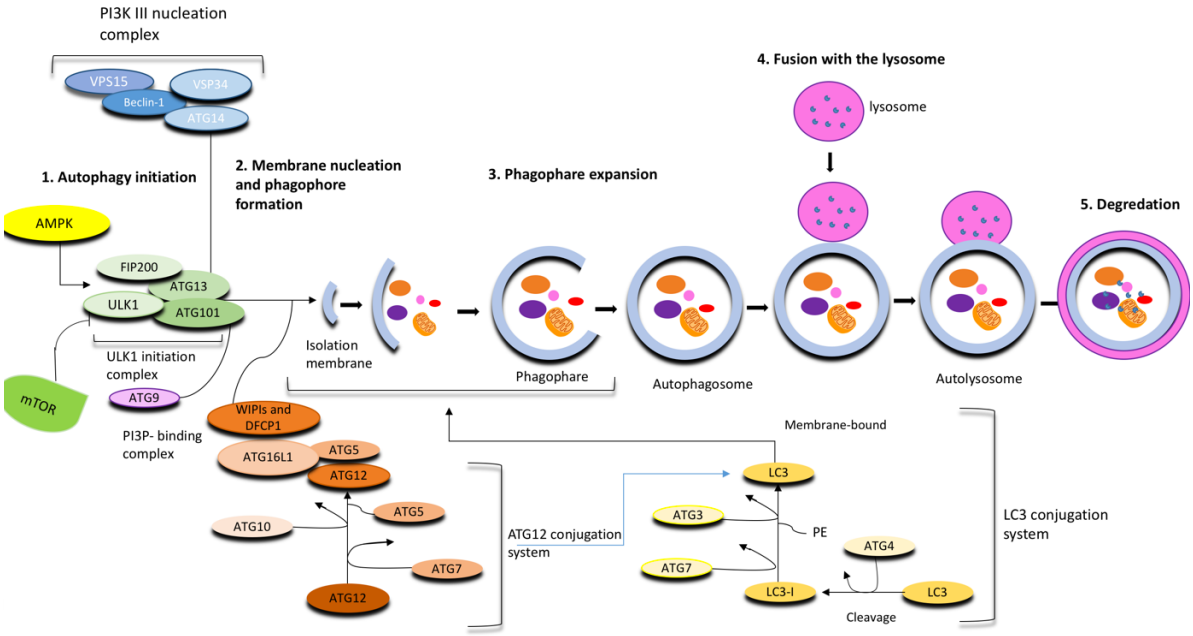

Figure 1.3.1.1 Molecular mechanisms of autophagy (Adapted from Hansen M, Rubinsztein DC, Walker DW. "Autophagy as a promoter of longevity: insights from model organisms.", Nat Rev Mol Cell Biol., 19, 579–593 (2018))

1.3.2 Regulation of Autophagy

Autophagy is a really complex process in which different proteins and pathways is involved [213]. The autophagic process is regulated by two main pathways: mammalian target of rapamycin complex 1 (mTORC1) and 5 adenosine monophosphate-activated protein kinase (AMPK). mTOR is a serine/threonine protein kinase which has been found in the phosphoinositol 3-kinase-related kinase (PI3K) protein family and has an important role in many essential cellular processes involving cell growth, survival and proliferation [219]. mTOR has two protein complexes: mTORC1 and mTORC2, but only mTORC1 has a role in direct regulation of autophagy [220].

mTORC1 regulates autophagy through inhibition of the ULK1 complex and this results in a dephosphorylated ULK1 complex. Thus, mTORC1 is a negative regulator of autophagy [221]. When mTOR is inhibited during the starvation, it leads to the activation of ULK1 and ULK2, which are phosphorylated and this results in the induction of autophagy. Another important regulator of autophagy involves the 5′ AMP-activated protein kinase (AMPK) and serine/threonine-protein kinase STK11 (LKB1). AMPK is the major energy sensor and its activation depends on the ATP ratio in the stress situation. During low energy conditions, AMPK is activated by LKB1 through inhibition of

mTORC1 thus it is a positive regulator of autophagy. In addition to that, AMPK could directly phosphorylate and activates the ULK1 [222], [223].

In addition to mTORC1 and AMPK, autophagy is regulated by many other signaling pathways. Interestingly, a study done by Jimenez-Sanchez et al. have shown that blocking of autophagy by Hh is controlled by the phosphorylated extracellular signal-regulated kinases – eukaryotic transcription factor alpha (pERK- eIF2α) [224]. Moreover, Milla et al. have shown that Hh directly regulates autophagy via the existence of multiple Gli consensus binding sites on the human ATG5 promoter. In addition, they have observed that autophagy activation is prevented due to Hh inhibition in neuroblastoma [225]. In addition, study done by Wang et al. shown that combination treatment with Hh pathway inhibitor GANT61 and autophagy inhibitor 3-MA decreased the cell viability in neuroblastoma cells [226]. Another study revealed that combination treatment of Chloroquine (CQ), which is an autophagy inhibitor, and GANT61 led to increasing cytotoxicity in hepatic stellate cells. [227]. Furthermore, Xu et al. showed that inhibition of Hh pathway with GANT61 induce the autophagic pathways in pancreatic ductal adenocarcinoma cells [228].

1.3.3 The role of the Autophagy in AML

Autophagy has crucial roles in many cellular processes and recent evidence demonstrates that it is associated with tumorigenesis, metastasis, and drug resistance in many cancers including pancreatic, breast, lung, colorectal, and leukemias [19-22]. The role of the autophagic pathway in cancer is controversial. Many studies have shown that depending on the cellular context, autophagy could act as a prosurvival or prodeath mechanism [229]. Previous studies have indicated that autophagy has an important role in the progression and pathogenesis of several subtypes of AML [22]. In silico analysis done by Watson et al. revealed that the decrease in the expression level of human Atg8 homologs autophagy-related genes including GAPARAPL1, GABARAPL2 and MAP1LC3B correlated with aberrant proliferation and development of AML blast [230]. Moreover, Jin et al. revealed that autophagy-related genes, which are composed of ULK1, FIP200, ATG14, ATG5, ATG7, ATG3, ATG4B, and ATG4D, exhibited low expression levels in AML patients' samples compared to healthy samples [231]. It is shown that autophagy has a role in the progression of myelodysplastic syndrome (MDS), which are comprised of the heterogeneous group of myeloid disorders, to AML [232-233]. Because

aberrant PI3K/AKT/mTORC1 axis is associated with the pathogenesis of AML, treatment with mTORC1 inhibitor, an autophagy suppressor, caused induction of autophagy in AML patients. Combination treatment with Chloroquine (CQ) and mTORC1 inhibitor lead to an increased cytotoxicity and this suggest that the combination of autophagy and mTORC1 inhibitors could prevent leukemic growth [234]. A similar study done by Willems et al. showed that mTOR kinase inhibitor, AZD8055, caused the induction of autophagy in AML transplanted mice and result in decreased proliferation of AML cells [235]. Moreover, Stankov et al. revealed that inhibition of autophagy under a critical threshold in combination with HDACis resulted in a decrease in the proliferation and survival of Down syndrome-associated megakaryoblastic leukemia (DS-AMKL) cells and patient's samples [236]. In addition, Nourkeyhani et al. reported that combination with Chloroquine (CQ) and Bafilomycin (BAF), autophagy inhibitors, and ARA-C, a chemotherapeutic agent, result in increased apoptosis and decreased cell proliferation in AML cells [237]. Also, Folkerts et al. reported that the use of hydroxychloroquine (HCQ), which is an autophagy inhibitor abrogated leukemic growth in the different subgroups of AML [238]. In another work done by Torgersen et al., they described the pro-survival effects of autophagy induction in AML-ETO samples. Combination treatment of the histone deacetylase inhibitors (HDACis) valproic acid (VPA) or vorinostat (SAHA) and an autophagy inhibitor CQ lead to induced apoptotic cell death in AML-ETO cells [239]. On the contrary, treatment with ATRA has a role in the degradation of PML-RARA fusion protein aggregates and result in enhanced autophagy in APL [240].

Hh signaling pathway was shown to crosstalk with other pathways and it regulates autophagy. The extensive crosstalk of this pathway with other pathways adds to its complexity and inhibiting it might not be a successful treatment option for cancer. Some clinical trials have shown that the Hh pathway inhibitors show unexpected results. For instance; the clinical study was done by Catenacci et al. combination of vismodegib, which is a Hh pathway inhibitor, and gemcitabine did not show any benefit in the pancreatic cancers (PCs) [241]. In addition to that, the Placebo-Controlled Study of Vismodegib did not show an improvement in the progression-free survival (PFS) of ovarian cancer patients [242]. Also, it is known that, hedgehog inhibition leads to the activation of autophagy [224], which in the context of AML, acts as a prosurvival mechanism. Thus, understanding the relationship between autophagy, Hh pathway and

other signaling pathways is important to overcome the leukemic growth. Thus, combination of Hh signaling and autophagy may be a more effective compared to single treatments in the prevention of AML leukemic growth. Therefore, understanding the importance of Hh and autophagy in the different subgroups of AML will allow us to administer a more targeted and effective therapy to combat AML.

Chapter 2

2. Materials and Methods

2.1 Cell Maintenance

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

- MOLM-13 AML M5 cell line with an FLT3-ITD mutation.
- CMK are AML M7 cell line from down-syndrome patients (DS- AMKL)

The cells were cultured in RPMI medium supplemented with FBS (10 % for MOLM-13 and 20% for CMK) and 100 U/mL penicilin/streptomycin at 37 ^{o}C in 5% CO_2. These cell lines were purchased from the German National Resource Center for Biological Material (DSMZ).

Confluent MOLM-13 cells were seeded out as 1.0 x 10^6 cells/ml and were split into 1:2 to 1:3 every 2 days. MOLM-13 cells were collected and centrifuged for 5 minutes at 800 rpm. The supernatant was discarded and the pellet was dissolved with fresh media. The cells seeded out into the 75 cm^2 sterile flask.

Confluent CMK cells were seeded out as 0.5 x 106 cells/ml and were split into 1:2 every 3 days. CMK cells were collected and the collected cells were centrifuged for 5 minutes at 700 rpm. The supernatant was discarded and the pellet was dissolved with fresh media. The cells seeded out into the 75 cm^2 sterile flask.

2.2 Detection of Protein Expression by Western Blotting

2.2.1 Protein Isolation

The cells were seeded into 6-well plates ($5x10^6$cells/mL) and treated with drugs for 48h. The cells were collected into the sterile falcon tubes and they were centrifuged at 1300 rpm for 5 min. The pellet was rinsed with 1 ml cold phosphate-buffered saline (PBS) and centrifuged at 1300 rpm for 5 min. This process was repeated twice. Then cells were lysed in 1X RIPA lysis buffer (ChemCruz, cat.no. sc-24948) containing 10µl protease inhibitor cocktail,10µl PMSF solution and 10µl sodium orthovanadate solution per ml. The cells were collected into the 2 ml eppendorf tube and pipetted with 21G syringe. Resuspended cells were vortexed and incubated on ice for at least 20 min. In the incubation time, the cells were vortexed for 5 min. After incubation, the cells were centrifuged at 4 ^{0}C max speed at least 20 min. with small cooling centrifuge. The supernanat was taken and was stored at -20 ^{0}C.

2.2.2 Determination of Concentration of Obtained Proteins

In order to determine the concentration of isolated proteins, DC protein assay kit (Biorad/USA cat. no. 500-0113, cat.no. 500-0114, cat. no. 500-0115) was used following manufacturer's instruction. The absorption was measured with a Varioskan™ LUX multimode microplate reader (Thermo Scientific™) at 750 nm.

2.2.3 Western Blotting

For western blotting, the whole cell lysates were loaded at a protein concentration of 30 µg per well. SDS-Gel electrophoreses (10-15% acrylamide gels based on the protein size) were performed into the 1X Running buffer at 100 V (Biorad, München). After running the gel, PVDF-membrane was first pre-activated 30 s in MeOH. After that the preactivated PVDF-membrane and after activation of whatman papers with 1X Transfer Buffer for 10 min (Biorad, München). After activation, the gel was transferred into the PVDF membrane for 20 min by using Trans-Blot Turbo Transfer System (Biorad, München). The membrane was blocked by using 5% dried milk in 10X TNT (5M NaCI, 2M Tris pH 7,5 and 10 % Tween20)

for 1 hr at room temperature. After blocking, the memrane was incubated overnight at 4 ^{0}C with the primary antibodies. The following antibodies were used: 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. After overnight incubation, the membrane was washed 3 times for 10 min on the rocking shaker. The membrane was incubated with the following secondary antibodies for 1hr at room temperature on the rocking shaker: 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). After 1hr incubation, membrane was washed 3 times for 10 min on the rocking shaker. The signals were detected by Pierce™ ECL Western Blotting Substrate (cat. no: 32106; UK) with ChemiDoc™ Imaging Systems (Biorad). Protein quantification was done by using Image Lab Software.

2.3 Drug Preparation and *In Vitro* Cell Viability Assay

For checking whether the hedgehog and autophagy pathways are activated in AML, the different AML cell lines were formed with different treatment groups:

- Dimethysuldoxide (DMSO)
- Hedgehog pathway inhibitors
- Autophagy pathway blockers
- Combination of Hedgehog and autophagy pathway inhibitors

Stock solutions of Gli inhibitor GANT61 was prepared by dissolving them in DMSO. Cells were treated with GANT61 (5μM-50μM) and were analyzed after 24, 48, and 72 hours to determine the IC50 concentrations. IC20, IC30, IC50 and IC80 values were determined by Graphpad prism 8 program based on the proliferation curve. Also, the autophagy pathway was manipulated at different levels by different autophagy blockers. Activation was achieved by using PP242 (0.01- 0.05μM). Inhibition of autophagy was achieved as follows: autophagosome-lysosome fusion using nocodazole (0.01 to 0.1 μM); autophagolysosomal degradation using NH_4Cl (0.5 to 10mM), chloroquine and hydroxychloroquine (5 to 100μM).

Cell proliferation was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) Cell Viability Assay. The cells were seeded in triplicate in 96-well plates at a plating density of 10^4 cells per each well at the 3 different time-points (24, 48, 72 h). 10 μl of MTT solution (cat. no. M2128; Sigma aldrich) was added to each well and the 96-well plates were incubated for 2h at 37°C. After 2h incubation, 96-well plates were centrifuged at the 1800 rpm for 10 min. Then, 100 μl of DMSO solution was added into the each well to solubilize the crystals and the plates were incubated for 15 min on the waving rotator. The absorbance was measured with a Varioskan™ LUX multimode microplate reader (Thermo Scientific™) at 570 nm.

2.4 Autophagy Modulation by Autophagy Blockers

The autophagy pathway was manipulated at different levels by different autophagy blockers. Autophagy manipulation was performed in accordance with the recently updated guidelines, which were published in Cell (Mizushima et al., 2010). Activation was achieved by using PP242 (0.01- 0.05μM). Inhibition of autophagy were achieved as follows: autophagosome-lysosome fusion using nocodazole (0.01 to 0.1 μM); autophagolysosomal degradation using NH_4Cl (0.5 to 10mM), chloroquine and hydroxychloroquine (5 to 100μM). Autophagic activity was measured in accordance with the recently updated guidelines (Mizushima et al., 2010). We checked the protein expression of the autophagy marker: LC3B. The following antibodies were used for Western blot analysis: LC3B, AKT, GAPDH and Anti-β-Actin and the above mentioned experimental procedure for western blotting was followed to detect the expression of these autophagic markers.

2.5 Cell Death Assay

For apoptosis assay, the cells were seeded into the 6 well plate for 48h (10^6cells/mL). After 48h incubation, the cells were collected and centrifugated at 1700 rpm for 5 minutes. Then, the cells were washed with phosphate-buffered saline (PBS) twice. After centrifugation, the cell pellets were dissolved in 200 μl binding buffer. To perform the measurement of apoptitc cells, 5 μL of fluorochrome-conjugated Annexin V (eBioscience™ Annexin V Apoptosis Detection Kit APC, cat. no. 88-8007-72) was added to each 200 μL sample and cell were incubated 10-15 min. at room temperature. After

incubation, the cells centrifugated at 1700 rpm for 5 minutes and were dissolved with 200 µL of 1X binding buffer. 5 µL of Propidium Iodide staining solution (eBioscience™ Annexin V Apoptosis Detection Kit APC, cat. no. 88-8007-72) was added and apoptosis was measured with BD LRFortessa^{TM} Cell Analyzer flow cytometer.

2.6 Combination Experiments

For the combination experiments IC20 and IC30 drug concentrations was determined based on proliferation curve. The combination experiments were performed according to Table 2.6.1.

Cells	Drugs and Dosages
MOLM-13	Chloroquine 6 µM +IC20-GANT61 5 µM
	Chloroquine 6 µM + IC30-GANT61 6 µM
	Nocodazole 0.03 µM +IC20-GANT61 5 µM
	Nocodazole 0.03 µM + IC30-GANT61 6 µM
CMK	GANT61 6 µM+IC20-NH_4CI 1 mM
	GANT61 6 µM+IC30-NH_4CI 2 mM
	Nocodazole 0.02 µM + IC20-GANT61 5 µM
	Nocodazole 0.02 µM + IC30-GANT61 6 µM

Table 2.6.1 Concentrations of drugs for combination experiments

2.8 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. The level of significance was set at $P<0.05$. All data were presented as mean ±s.d. Calculations were performed by using GraphPad Prism 8.

Chapter 3

3. Results

To understand the crosstalk between autophagy and Hh signaling pathways, two AML cell lines, MOLM-13, and CMK, were selected for all experiments. These cell lines were treated with either autophagy modulators or Hh inhibitor and their combinations at the different time points based on the experiments. All experiments were performed on these cells in vitro.

3.1. The Effect of the Manipulation of Autophagy on the proliferation of AML Cell Lines

Many studies have shown that depending on the cellular context autophagy could act as a suppressor or an activator in cancer development [243-246]. Because of this dual role of autophagy in cancer and in order to understand the role of autophagy on our cell lines, we have used different autophagy modulators. The manipulation of autophagy could be achieved by either the use of autophagy activators or inhibitors. One way to activate autophagy is to target the mTOR pathway, a negative regulator of autophagy, and mTOR inhibitors are used for this purpose [226]. One of these inhibitors is PP242, which is an ATP‑competitive inhibitor of mTOR (mammalian target of rapamycin) kinase. Because mTOR is a negative regulator of autophagy, PP242 treatment leads to the induction of autophagy [247-249]. In our study, in order to show effect of autophagy activation on the proliferation of AML cell lines, we treated our cells with PP242. MOLM-13 and CMK cells were treated with PP242 at different concentrations (0.01-0.05 μM) and time points (24, 48 and 72h). We found that the proliferation of MOLM-13 cells (Figure 3.1.1.a) did not change compared to the control and we actually saw an increase in the proliferation of these cells at 48h at 0.02, 0.04 and 0.05 μM of PP242 treatment. The proliferation of CMK cells (Figure 3.1.1.b) did not significantly change at the highest concentration of PP242 (0.05 μM) compared to control. This suggests that activation of autophagy has no effect on CMK cells and led to a slight increase in the proliferation of MOLM-13 cells, which suggest that chemical activation of autophagy has no significant effect on the survival of these cells.

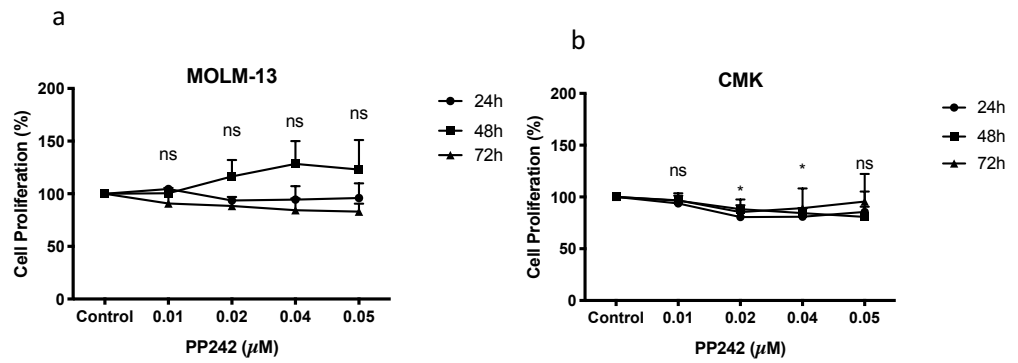

Figure 3.1.1 The effect of PP242 treatment on the proliferation of MOLM-13 (a) and CMK (b) cells after 24, 48 and 72 h. Normalization was done per control and shown as percentage of proliferation cells. 3 independent experiments were performed and each experiment consisted of 3 replicate. The significance of the test was evaluated as p <0.05 by using GraphPad Prism 8 (n=3). (ns=P > 0.05, * =P ≤ 0.05 , **= P ≤ 0.01=, *** P ≤ 0.001, **** =P ≤ 0.0001)

Many studies reported that autophagy acts as a promoter of cancer growth and the targeting of the autophagic pathway with specific inhibitors could prevent tumor growth [250-252]. We inhibited autophagy either at the autophagosome-lysosome fusion step using nocodazole (0.01 to 0.1 μM) or at the autophagolysosomal degradation using Ammonium chloride, Chloroquine or Hydroxychloroquine, which are the lysosomotropic reagents.

Ammonium Chloride is a well-known autophagy inhibitor, which prevents the fusion of lysosome to the autophagosome [251,253]. After the treatment of MOLM-13 cells with Ammonium Chloride (0.5 mM and 10 mM), we observed that the proliferation of MOLM-13 cells (Figure 3.1.2.a) was significantly decreased by 80-90% at the highest concentration (10 mM) compared to control at 48h. On the other hand, CMK cells (Figure 3.1.2.b) showed a %50 decrease compared to the control at the highest dosage of Ammonium Chloride at 48h (10 mM).

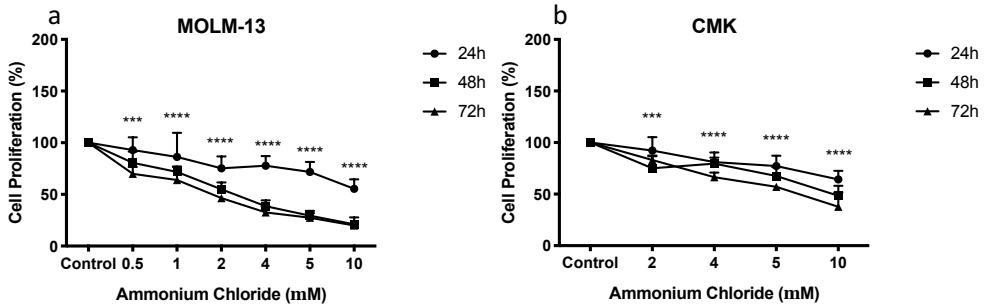

Figure 3.1.2 The effect of Ammonium Chloride treatment on the proliferation of MOLM-13 (a) and CMK (b) cells after 24, 48 and 72 h. Normalization was done per control and shown as percentage of proliferation cells. 3 independent experiments were performed and each experiment consisted of 3 replicate. The significance of the test was evaluated as p <0.05 by using GraphPad Prism 8 (n=3). (ns=P > 0.05, * =P ≤ 0.05, **= P ≤ 0.01=, *** P ≤ 0.001, **** =P ≤ 0.0001)

To further confirm the effect of the inhibition of autophagy at the autophagolysosomal degradation, Chloroquine (CQ) and Hydroxychloroquine (HCQ) drugs were used. The treatment with CQ and HCQ leads to an increase in the lysosomal pH level and this prevents the fusion of the lysosome to the autophagosome. [251-252]. In order to check the effect of CQ and HCQ on the proliferation of MOLM-13 and CMK cell lines, we treated the cells with these drugs. After treatment of the MOLM-13 with CQ (5-100 μM) and HCQ (5-100 μM), we observed that the proliferation of MOLM-13 cells decreased by 80-90% upon the treatment with either CQ or HCQ for 48h at 100 μM (Figure 3.1.3.a, Figure 3.1.4.a, respectively). Similarly, it is observed that the proliferation of CMK cell line decrease by %70 after the treatment with both drugs (Figure 3.1.3.b, Figure 3.1.4.b, respectively).

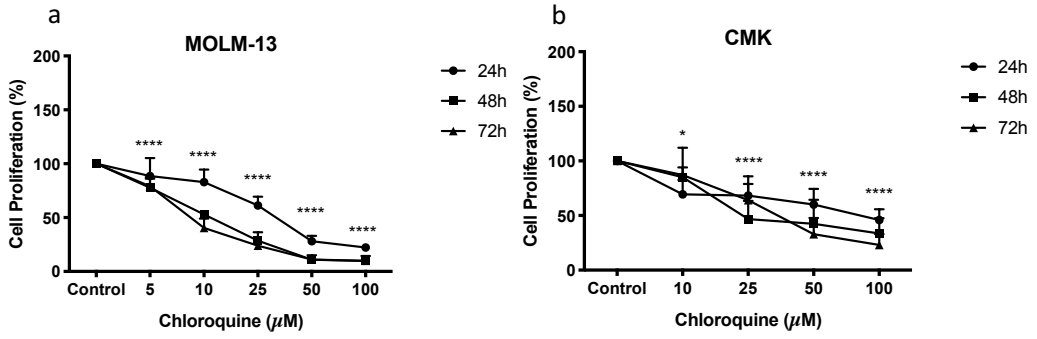

Figure 3.1.3 The effect of Chloroquine (CQ) treatment on the proliferation of MOLM-13 (a) and CMK (b) cells after 24, 48 and 72 h. Normalization was done per control and shown as percentage of proliferation cells. 3 independent experiments were performed and each experiment consisted of 3 replicate. The significance of the test was evaluated as p <0.05 by using GraphPad Prism 8 (n=3). (ns=P > 0.05, * =P ≤ 0.05, **= P ≤ 0.01=, *** P ≤ 0.001, **** =P ≤ 0.0001)

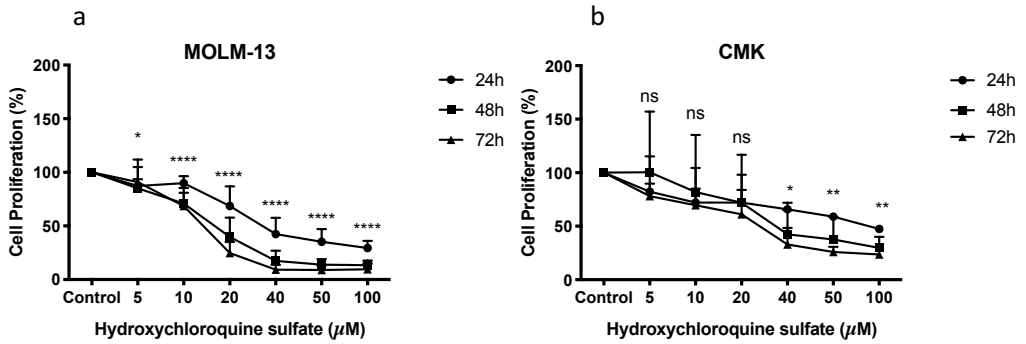

Figure 3.1.4 The effect of Hydroxychloroquine (HCQ) treatment on the proliferation of MOLM-13 (a) and CMK (b) cells after 24, 48 and 72 h. Normalization was done per control and shown as percentage of proliferation cells. 3 independent experiments were performed and each experiment consisted of 3 replicate. The significance of the test was evaluated as p <0.05 by using GraphPad Prism 8 (n=3). (ns=P > 0.05, * =P ≤ 0.05, **= P ≤ 0.01=, *** P ≤ 0.001, **** =P ≤ 0.0001)

Nocodazole is a microtubule-depolymerizing agent and has a role in the inhibition of autophagy via the disruption of the activity of microtubules [254]. Nearly, cell viability of both cells diminished by 80-90 % after the Nocodazole treatment at 48h (Figure 3.1.5).

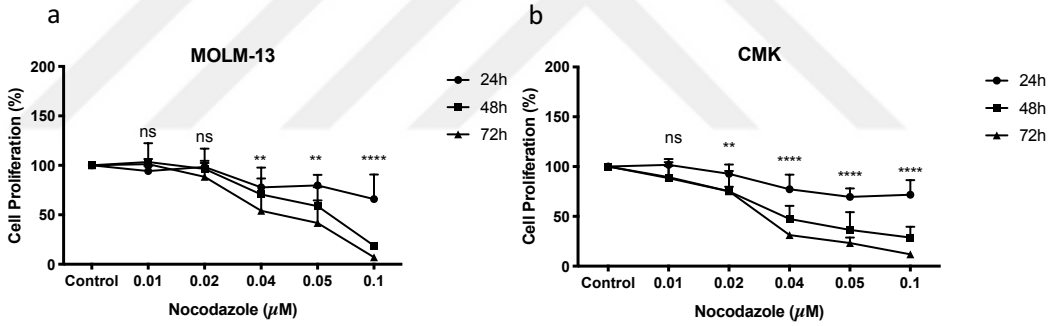

Figure 3.1.5 The effect of Nocodazole treatment on the proliferation of MOLM-13 (a) and CMK (b) cells after 24, 48 and 72 h. Normalization was done per control and shown as percentage of proliferation cells. 3 independent experiments were performed and each experiment consisted of 3 replicate. The significance of the test was evaluated as p <0.05 by using GraphPad Prism 8 (n=3). (ns=P > 0.05, * =P ≤ 0.05, **= P ≤ 0.01=, *** P ≤ 0.001, **** =P ≤ 0.0001)

All together, these results showed that inhibition of autophagy diminished the proliferation of AML cell line. This suggests the role of autophagy as a prosurvival mechanism in the context of AML.

3.2 Treatment of AML Cell line with Hedgehog Pathway Inhibitor Affects the Proliferation of AML Cell Lines

Many studies showed that the aberrant Hh pathway has an important role in the pathogenesis of different types of cancer including hematopoietic malignancies [255-256]. Therefore, targeting this pathway with special Hh inhibitors could prevent leukemic growth. Since the FDA approved Glasdegib, which is a small molecule inhibitor of Smoothened (SMO), for AML treatment in 2018, researchers started to have a new-found interest in Hh signaling inhibitors for the treatment of AML [257]. Moreover, targeting GLI transcription factors with specific inhibitors presents a new therapeutic approach for the treatment of AML. One of these inhibitors is GANT61, which is the first GLI antagonist, it inhibits GLI1 and GLI2 transcription factor [179, 258-260].

In our study, we wanted to check the effect of the inhibition of the hedgehog pathway, for that we treated our cells with GANT61 and checked its effect on the proliferation of MOLM-13 and CMK cells. Dosage of GANT61 was optimized as 5-50 μM for MOLM-13 cells (Figure 3.2.1.a) and 5-20 μM for CMK cell line (Figure 3.2.1.b). When we treated MOLM-13 cell line with GANT61 in both time and dose-dependent manner, we observed that the proliferation of these cells decreased approximately by %95. On the other hand, CMK cells decreased by %50 at the highest dosage (20 μM).

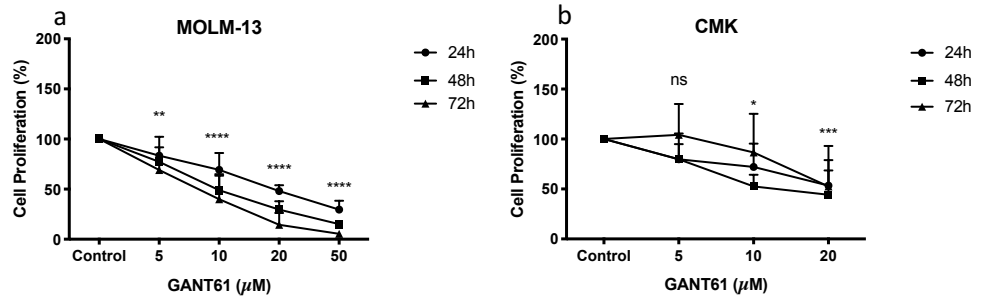

Figure 3.2.1 The effect of GANT61 treatment on the proliferation of MOLM-13 (a) and CMK (b) cells after 24, 48 and 72 h. Normalization was done per control and shown as percentage of proliferation cells. 3 independent experiments were performed and each experiment consisted of 3 replicate. The significance of the test was evaluated as p <0.05 by using GraphPad Prism 8 (n=3). (ns=P > 0.05, * =P ≤ 0.05, **= P ≤ 0.01=, *** P ≤ 0.001, **** =P ≤ 0.0001)

3.3 The Effect of Combination Treatment with Hh Pathway and Autophagy Pathway Inhibitors on the Proliferation of MOLM-13 and CMK Cell Lines.

In order to show the synergistic, antagonistic or additive effect of combination treatment on the proliferation of MOLM-13 and CMK cells, we used different autophagy inhibitors and the Hh pathway inhibitor, GANT61. Firstly, we determined the IC20 and IC30 values of drugs for combination therapy (Table 2.6.1). Once the IC20 and IC30 values were determined, we initiated the combination treatments of autophagy pathway inhibitors and GANT61 Hh inhibitor on MOLM-13 and CMK cell lines.

In order to check the synergistic, antagonistic or additive effect of autophagy and Hh inhibitors for MOLM-13, we performed two different combination treatments. Firstly, we used GANT61 as a Hh inhibitor and CQ as an autophagolysosome degradation inhibitor (Figure 3.3.1.a). We fixed the CQ concentration on the IC30 value (6 uM) and we changed GANT61 dosage (Chloroquine 6 μM +IC20-GANT61 5 μM, Chloroquine 6 μM + IC30-GANT61 6 μM). At the end of the two combinations, we observed that the proliferation of MOLM-13 cells significantly decreased compared to the DMSO control, CQ alone (6 μM) and GANT61 alone (5 μM and 6 μM) for 48h. Based on the proliferation results and upon comparing the proliferation bars of GANT61 and Chloroquine combination treatment to single treatments, the combination treatment exhibited a synergistic effect in MOLM-13 cells. After that, we used GANT61 as Hh inhibitor and Nocodazole as a microtubule-depolymerizing autophagy inhibitor (Figure 3.3.1.b). For combination experiment, we fixed the Nocodazole concentration on the IC30 value (0.03 μM) and we changed GANT61 dosage (Nocodazole 0.03 μM +IC20-GANT61 5 μM, Nocodazole 0.03 μM + IC30-GANT61 6 μM) for 48h. At the end of the two combinations, we observed that the proliferation of MOLM-13 cells significantly decreased to a value below %50 compared to the DMSO control, Nocodazole alone (0.03 μM) and GANT61 alone (5 μM and 6 μM). Depending on our proliferation results and upon comparing the proliferation bars of GANT61 and Nocodazole, we can conclude that combination treatment showed a synergistic effect on MOLM-13 cell line.

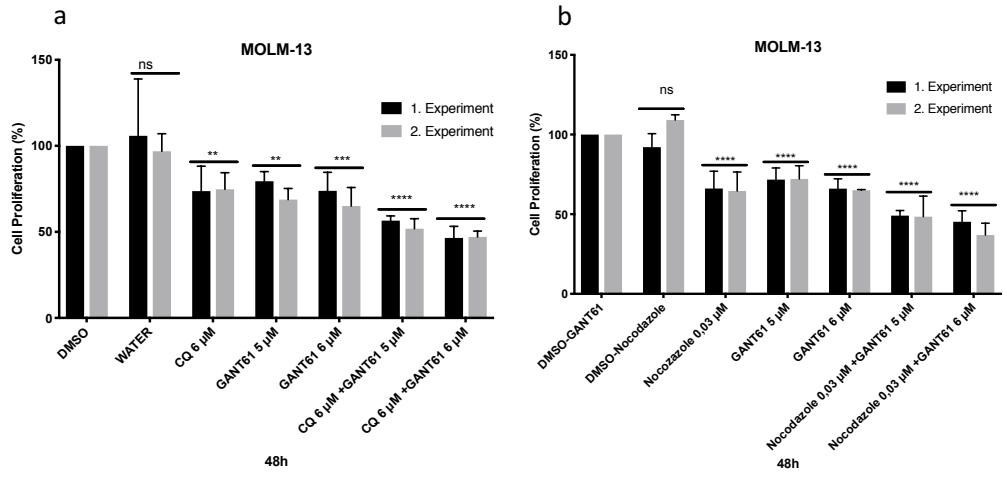

Figure 3.3.1 (a) The effect of GANT61 and Chloroquine combination treatment on the proliferation of MOLM-13 cells after 48h (n=2). (b) The effect of GANT61 and Nocodazole combination treatment on the proliferation of MOLM-13 cells after 48h (n=2). (a-b) Normalization was done per control and shown as percentage of proliferation cells. Each experiment consisted of 3 replicate. 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)

In order to reveal the synergistic, antagonistic or additive effect of autophagy and Hh inhibitors for CMK, we performed two different combination treatments. Firstly, we used GANT61 as Hh inhibitor and NH_4CI as lysosomal autophagy inhibitor (Figure 3.3.2). We fixed the GANT61 concentration on the IC30 value (6 uM) and we changed NH_4CI dosage (GANT61 6 µM+ NH_4CI 1 mM, GANT61 6 µM+IC20 NH_4CI 2 mM) for 48h. At the end of the two combinations, although we observed a decrease in cell proliferation compared to the DMSO control, the proliferation of CMK cells actually increased compared to GANT61 treament alone (6 µM) and NH_4CI treatment alone (1 mM and 2 mM). The results indicated that GANT61 and NH_4CI combination therapy exhibited antagonistic effect on CMK cell line.

Secondly, we used GANT61 as Hh inhibitor and Nocodazole as microtubule-depolymerizing autophagy inhibitor. We fixed the Nocodazole concentration on the IC30 value (0.02 µM) and we changed GANT61 dosage (Nocodazole 0.02 µM + GANT61 5 µM, Nocodazole 0.02 µM + GANT61 6 µM) for 48h. At the end of the two combinations, we observed that proliferation of CMK cells decreased compared to the DMSO control and Nocodazole alone (0.03 µM). On the other hand, proliferation of CMK cells increased compared GANT61 alone treatments (5 µM and 6 µM).

Consequently, autophagy and Hh pathway combination therapy exhibited synergistic effect on MOLM-13 cells compared to single treatments. However, autophagy and Hh pathway combination treatments resulted in antagonistic effect compared to single treatment of GANT61 in CMK cell line.

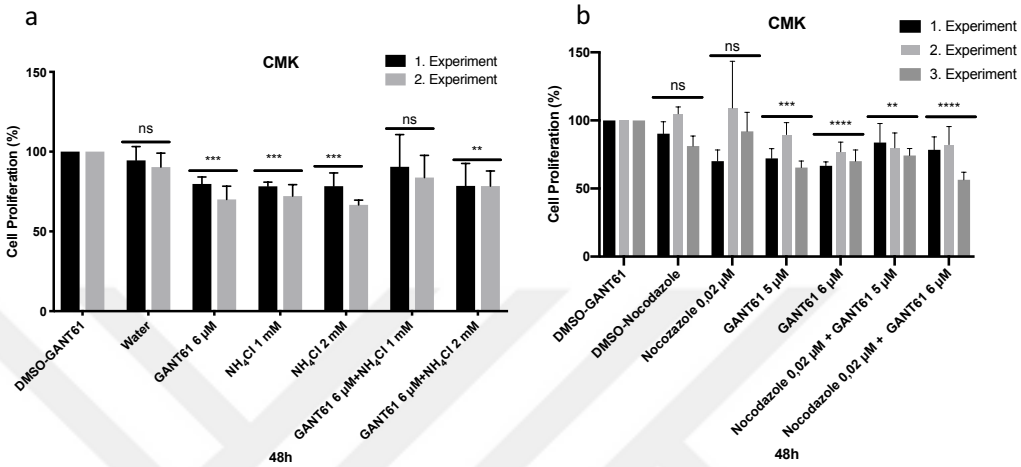

Figure 3.3.2 (a) The effect of GANT61 and NH_4CI combination treatment on the proliferation of CMK cells after 48h (n=2). (b) The effect of GANT61 and Nocodazole combination treatment on the proliferation of CMK cells after 48h. (a-b) Normalization was done per control and shown as percentage of proliferation cells (n=3). Each experiment consisted of 3 replicate. 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)

3.4 The Effect of Autophagy Inhibition on Cell Death of MOLM-13 and CMK Cell Lines

Apoptosis and autophagy have been found to be highly connected and that they actually regulate each other [261]. Many studies reported that autophagy inhibitors such as ammonium chloride, chloroquine or hydroxychloroquine affected cell death mechanisms [262-264]. To further study the role of autophagy in triggering cell death in our AML cell lines, we detected apoptotic and necrotic cell death using flow cytometric analysis after treatment with autophagy inhibitors. In order to determine the percentage of the necrotic and apoptotic cells, we treated them with different autophagy inhibitors. Upon the treatment of MOLM-13 cells with IC50 (2 mM) and IC80 (10 mM) concentration of ammonium chloride for 48h, the percentage of apoptotic cells didn't change. However, the percentage of necrotic cells increased by approximately 4 folds

compared to water control (Figure 3.4.1.a). After that, we treated the MOLM-13 cells with IC50 (10 μM) and IC80 (30 μM) concentrations of CQ for 48h. After treatment, both the percentage of apoptotic cells and necrotic cells increased at the IC80 value compared to the water control (Figure 3.4.1.b). On the other hand, when the MOLM-13 cells were treated 15μM and 30 μM of Hydroxychloroquine, it is observed that there is no change at the apoptotic cell percentage compared to the control (Figure 3.4.1.c). Similarly, we didn't see an apparent change on apoptotic or necrotic cell death after treatment with autophagy inhibitors on CMK cell line. (Figure 3.4.2.a/b). In conclusion, although CQ treatment in MOLM-13 cell increases both apoptosis and necrosis, inhibiting the autophagic pathway does not affect cell death on CMK depending on our data.

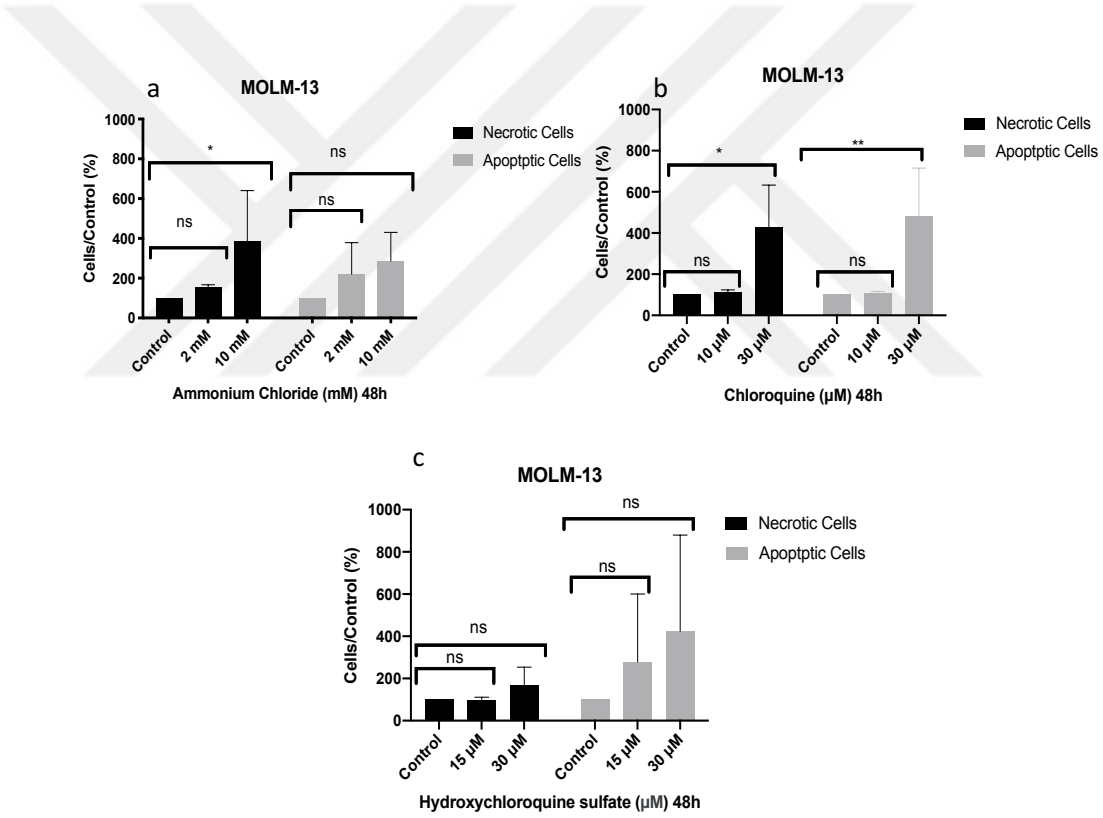

Figure 3.4.1 The effect of Ammonium Chloride (a), Chloroquine (b) and Hydroxychloroquine (c) on necrotic and apoptotic cell death of MOLM-13 cells after treatment for 48h. The data represents the quantification of necrotic and apoptotic cell percentage of MOLM-13 cells per water control. 3 independent experiments were performed. The significance of the test was evaluated as p <0.05 by using GraphPad Prism 8 (n=3). (ns=P > 0.05, * =P ≤ 0.05, **= P ≤ 0.01=, *** P ≤ 0.001, **** =P ≤ 0.0001)

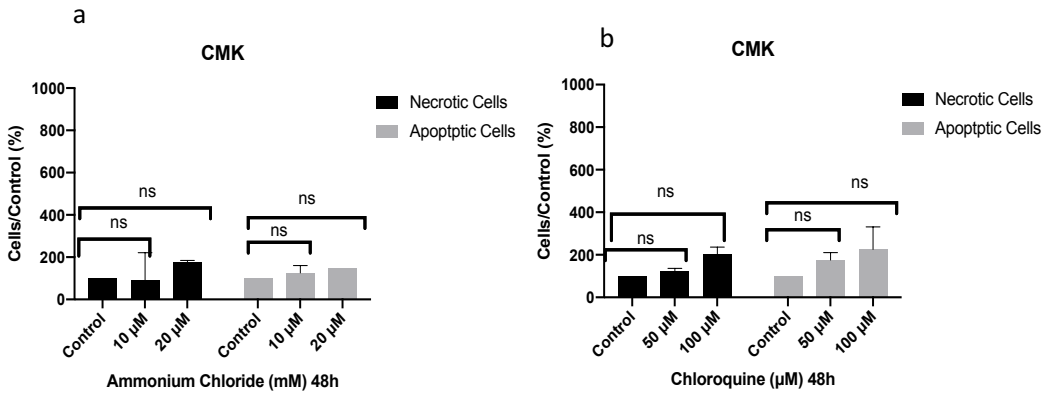

Figure 3.4.2 The effect of Ammonium Chloride (a) and Chloroquine (b) on necrotic and apoptotic cell death of CMK cells after treatment for 48h. The data represents the quantification of necrotic and apoptotic cell percentage of CMK cells per water control. 2 independent experiments were performed. The significance of the test was evaluated as p <0.05 by using GraphPad Prism 8 (n=2). (ns=P > 0.05, * =P ≤ 0.05, **= P ≤ 0.01=, *** P ≤ 0.001, **** =P ≤ 0.0001)

3.5 The Effect of Autophagy Modulation and the Combination of Hh and Autophagy Inhibitors on the Molecular Markers of the Autophagy

In order to understand the effect of autophagy and hedgehog treatment, we sought to look at the molecular markers of autophagy such as the expression of the microtubule-associated protein light chain 3 (LC3) I conversion to LC3-II. [251, 265]. Upon the fusion of lysosome and autophagosome, LC3B-II is degraded by lysosomal enzymes which leads to a decrease in the expression level of LC3B-II [251]. However, inhibition of autophagolysosomal degradation with lysosomotropic reagents such as ammonium chloride, Chloroquine or Hydroxychloroquine leads to accumulation of LC3B-II [265]. To understand this effect, we performed western blot assays on whole cell lysates. We checked the expression of LC3B proteins and wanted to check effect of this inhibition on AKT protein, a negative regulator of autophagy. For this, we used the IC50 and IC80 values for the single treatments of autophagy inhibitors. When we treated the MOLM-13 cells with NH_4CI for 48h, we observed that the expression of AKT protein did not change compared to water control. On the other hand, LCB3 expression, especially LC3B-II slightly increased in the MOLM-13 cells after treatment with NH_4CI, which suggests a block in autophagy that leads to an accumulation of LC3BII. After that, we wanted to check the effect of CQ on the protein expression level of AKT and LC3BII in MOLM-13 cells. We observed that the AKT level slightly decreased while the level of

L3CB-II increased in MOLM-13 cells after treatment with CQ (Figure 3.5.1). Due to the fact that Ammonium Chloride and CQ inhibited the autophagy pathway at the autophagolysosome-degradation step, we found that this treatment leads to an accumulation of LC3B-II.

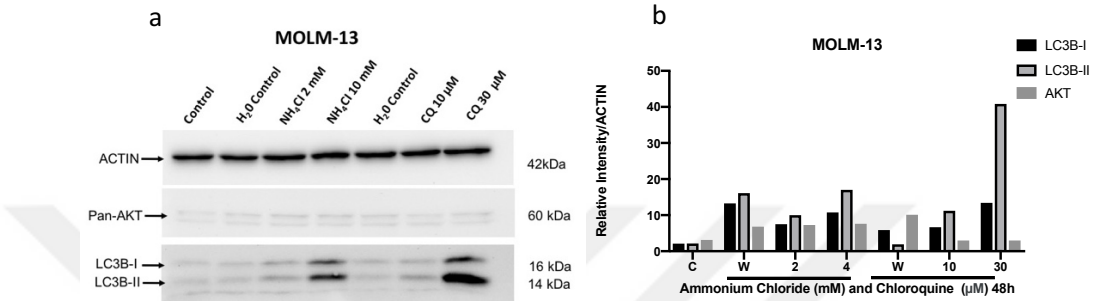

Figure 3.5.1. (a) The effect of NH_4CI and Chloroquine treatment on the expression level of AKT and LC3B proteins in MOLM-13 cells for 48h. (b) Graphical presentation shows the relative intensity of AKT and LC3B after normalization with Actin.

When the MOLM-13 cells were treated with hydroxychloroquine for 48h, we observed that the expression of LC3B-II protein increased. This results shows the successful inhibition in autophagy after HCQ treatment due to the accumulation in LC3B-II. In addition, AKT level slightly increased after treatment with HCQ in the MOLM-13 cells (Figure 3.5.2).

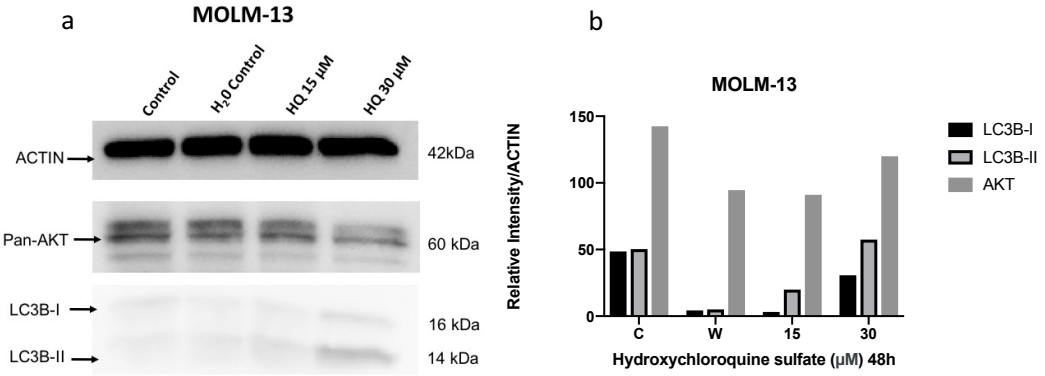

Figure 3.5.2 (a) The effect of HQ on the expression level of AKT protein in MOLM-13 cells for 48h (b) Graphical presentation shows the relative intensity of AKT and LC3B after normalization with Actin.

To check the effect of autophagy activation on the molecular markers of autophagy, PP242 which is an ATP‑competitive inhibitor of mTOR kinase, was used. It is a known activator of the autophagy [247- 249]. We treated the MOLM-13 cells with PP242 checked the effect of this treatment on the expression level of AKT protein for 48h. We observed that expression of AKT protein decreased compared to DMSO control. We can suggest that inhibition of the mTOR pathway with PP242 leads to a decrease of AKT protein.

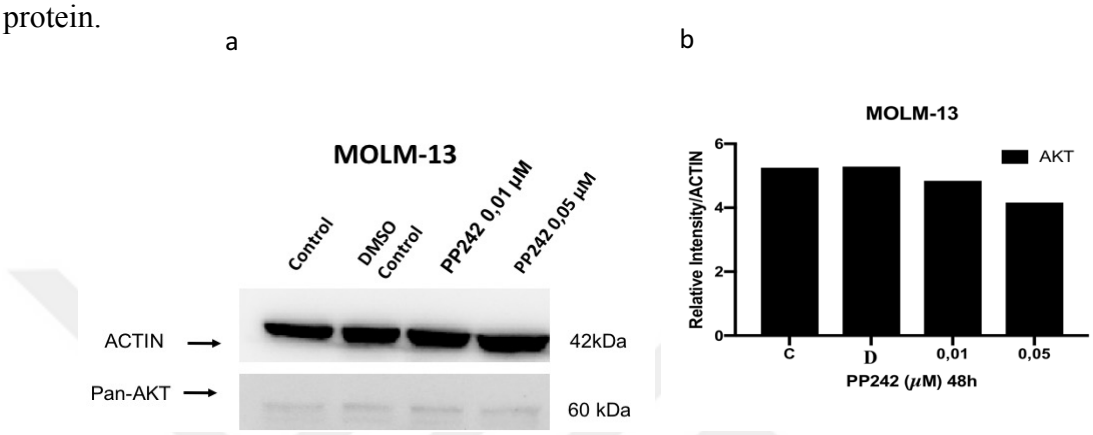

Figure 3.5.3 (a) The effect of PP242 on the expression level of AKT protein in MOLM-13 cells for 48h (b) Graphical presentation shows the relative intensity of AKT after normalization with Actin.

When we treated the MOLM-13 cells with Nocodazole for 48h, we observed that the expression of AKT protein diminished compared to DMSO control. Furthermore, the expression level of LC3B-I increased compared to control with Nocodazole treatment. On the other hand, although the expression of L3CB-II increased at the 0.05 μM concentration, it decreased at the dose of 0.1 μM after treatment with nocodazole.

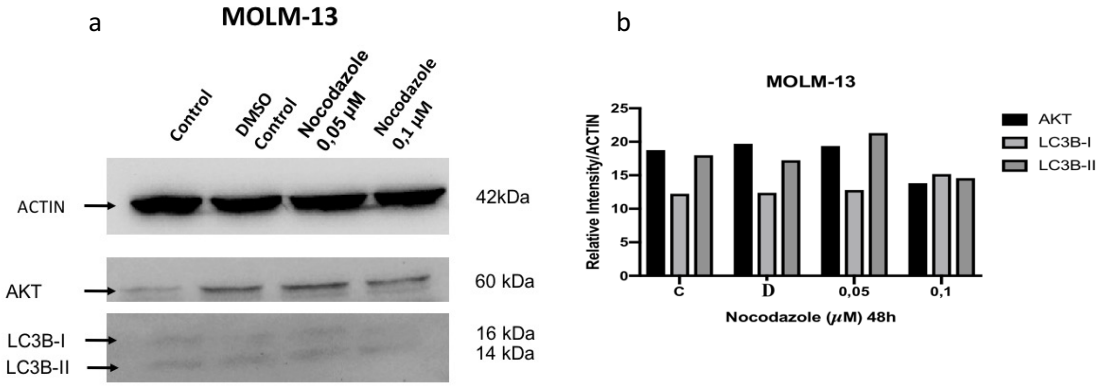

Figure 3.5.4 (a) The effect of Nocodazole treatment on the expression level of AKT and LC3B proteins in MOLM-13 cells for 48h. (b) Graphical presentation shows the relative intensity of AKT and LC3B after normalization with Actin.

After that, we treated the CMK cells in order to check the protein expression level of AKT and LC3B proteins with NH_4CI for 48h. We observed that expression of AKT protein slightly decreased compared to water control. On the other hand, LCB3 the expression, both LC3B-I and LC3B-II significantly increased in the CMK cells after treatment with NH_4CI (Figure 3.5.5). After that, we wanted to check the effect of CQ on the protein expression level in the CMK cells. It is observed that AKT level decreased and L3CB-I and L3CB-I significantly increased in CMK cells after treatment with CQ (Figure 3.5.5). Our results suggested that inhibition of autophagy with both Ammonium chloride and CQ treatments lead to the accumulation of LC3B in the CMK cell line.

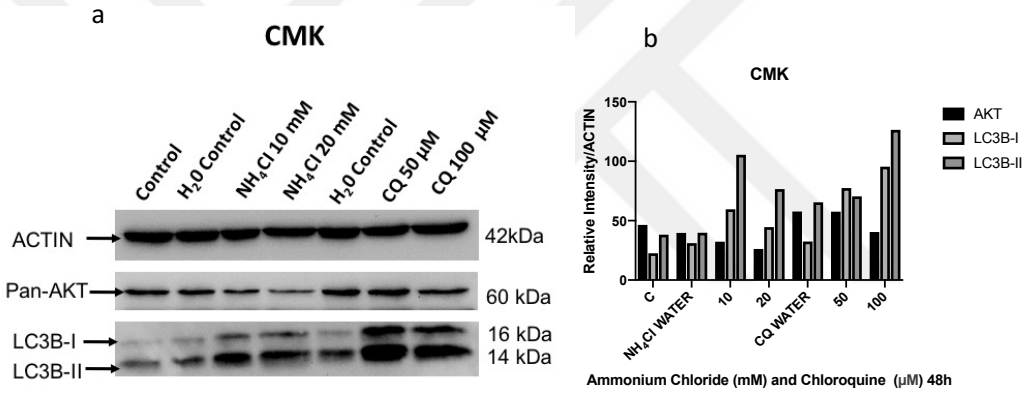

Figure 3.5.5 (a) The effect of Ammonium Chloride (NH_4CI) and CQ treatment on the expression level of AKT and LC3B proteins in CMK cells for 48h (b) Graphical presentation shows the relative intensity of AKT and LC3B after normalization with Actin.

To further confirm the effect of NH4Cl we used HCQ, another inhibitor of the autophagosome degradation step. When CMK cells were treated with hydroxychloroquine for 48h, we observed that the expression of LC3B-I and LC3B-II protein significantly increased. On the other hand, AKT level slightly decreased after treatment with HCQ in the CMK cells (Figure 3.5.6). Consistent with the literature, we can propose that HCQ treatment leads to the accumulation of LC3B-II, thus a block in autophagy.

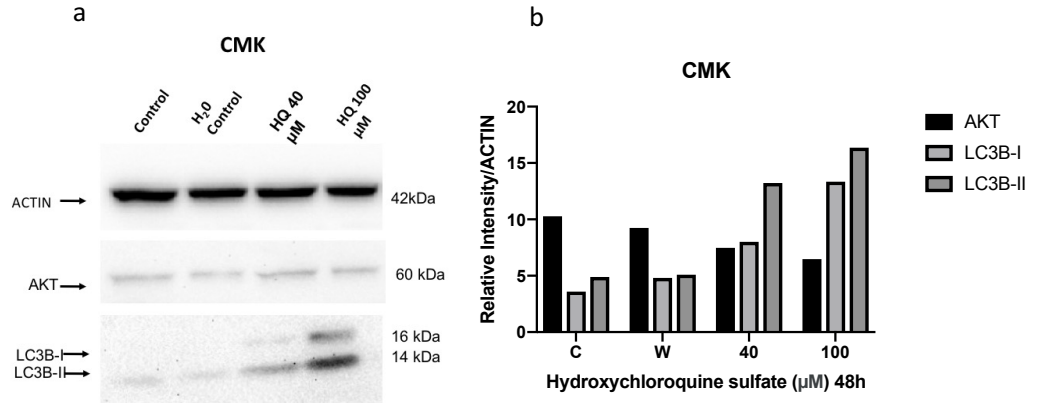

Figure 3.5.6 (a) The effect of HCQ treatment on the expression level of AKT and LC3B proteins in CMK cells for 48h (b) Graphical presentation shows the relative intensity of AKT and LC3B after normalization with Actin.

Previous studies have suggested that Hh signaling pathway has a role in the suppression of the autophagy [214, 266]. Therefore, Hh pathway inhibition could lead to the induction of autophagy. To understand the role of Hh inhibition on autophagy, we checked AKT and LC3B, autophagic marker. In order to do that, we performed different combination treatments of autophagy inhibitors and Hh pathway inhibitor, GANT61 on MOLM-13 and CMK cells.

Firstly, we used GANT61 as Hh inhibitor and CQ, as a lysosomal autophagy inhibitor (Figure 3.5.7). For combination experiment, we fixed the CQ concentration on the IC30 value (6 uM) and we changed GANT61 dosage (Chloroquine 6 µM +IC20-GANT61 5 µM, Chloroquine 6 µM + IC30-GANT61 6 µM) for 48h. We observed that the AKT level slightly increased after combination treatment compared to DMSO control in the MOLM-13 cells. Based on these results, we can propose that combination of GANT61 and Chloroquine led to increasing AKT expression for the MOLM-13 cell line.

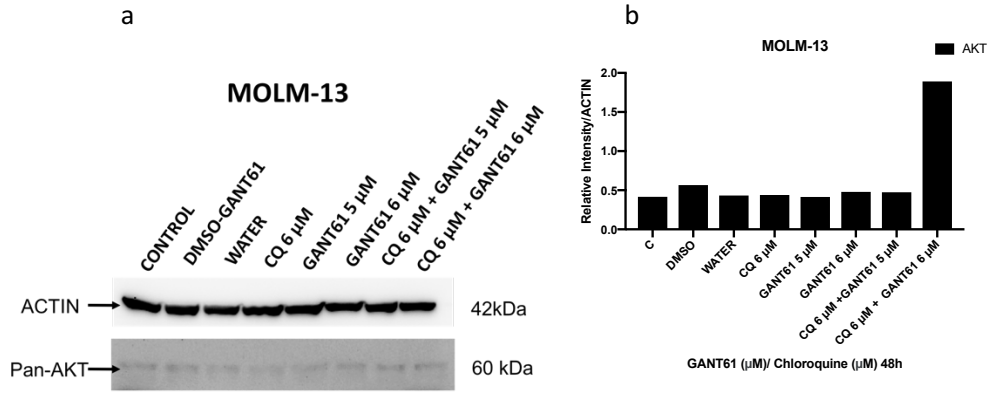

Figure 3.5.7 (a) The effect of GANT61/CQ combination tretment on the expression level of AKT protein in MOLM-13 cells for 48h (b) Graphical presentation shows the relative intensity of AKT after normalization with Actin.

After that, we used GANT61 as Hh inhibitor and Nocodazole as a microtubule-depolymerizing autophagy inhibitor (Figure 3.5.8). For combination experiment, we fixed the Nocodazole concentration on the IC30 value (0.03 µM) and we changed GANT61 dosage (Nocodazole 0.03 µM +IC20-GANT61 5 µM, Nocodazole 0.03 µM + IC30-GANT61 6 µM) for 48h. We observed that the expression level of AKT protein decreased and LC3B (I-II) protein significantly increased in MOLM-13 cells after combination treatment compared to control (DMSO-GANT61). GANT61 and Nocodazole combination treatments led to the accumulation of LC3B for MOLM-13 cells.

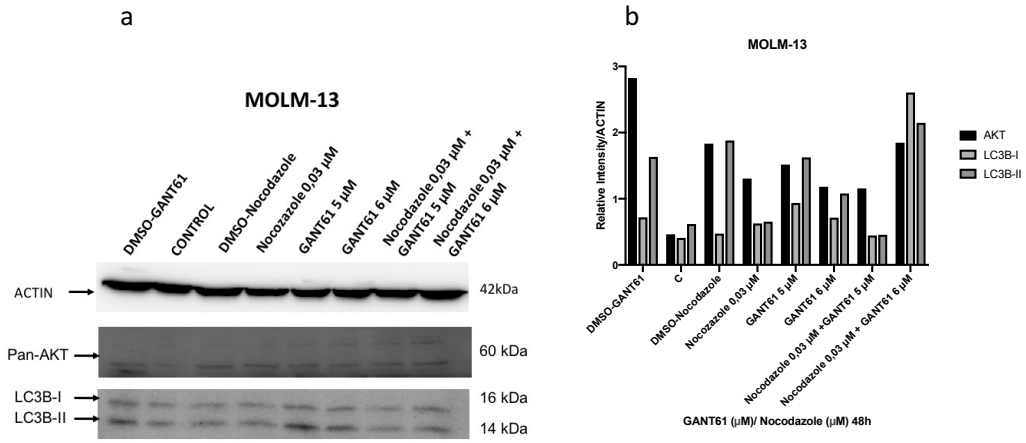

Figure 3.5.8 (a) The effect of GANT61/Nocodazole combination treatment on the expression level of AKT and LC3B proteins in MOLM-13 cells for 48h (b) Graphical presentation shows the relative intensity of AKT and LC3B after normalization with Actin.

For CMK cells, firstly, we used GANT61 as Hh inhibitor and NH_4CI as a lysosomal autophagy inhibitor (Figure 3.5.9). For combination experiment, we fixed the GANT61 concentration on the IC30 value (6 uM) and we changed NH_4CI dosage (GANT61 6 µM+ IC20-NH_4CI 1 mM, GANT61 6 µM+IC30 NH_4CI 2 mM) for 48h. We observed that the AKT level slightly decreased after combination treatment compared to DMSO-control in the CMK cells. The level of LC3B-II decreased after combination treatment compared to DMSO control. Depending on our data, GANT61 and NH4CI combination treatments led to a decrease in the expression level of both LC3B-II and AKT in CMK cells.

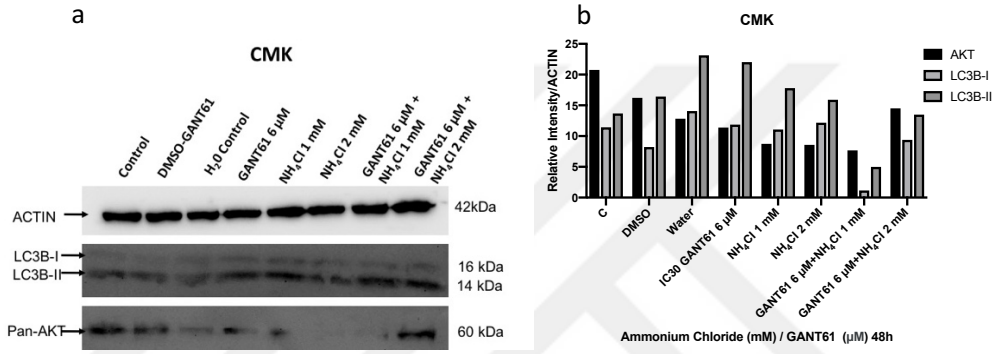

Figure 3.5.9 (a) The effect of GANT61/NH_4CI combination treatment the expression level of AKT and LC3B proteins in CMK cells for 48h (b) Graphical presentation shows the relative intensity of AKT and LC3B after normalization with Actin.

After that, we used GANT61 as Hh inhibitor and Nocodazole as a microtubule-depolymerizing autophagy inhibitor (Figure 3.5.10). For combination experiment, we fixed the Nocodazole concentration on the IC30 value (0.02 µM) and we changed GANT61 dosage (Nocodazole 0.02 µM + IC20-GANT61 5 µM, Nocodazole 0.02 µM + IC30-GANT61 6 µM) for 48h. Western blot analysis showed that the expression level of AKT and LC3B (I-II) proteins increased in CMK cells after combination treatment compared to control (DMSO-GANT61). We can suggest that the combination of GANT61 and Nocodazole led to both AKT and LC3B-II accumulation on CMK cells.

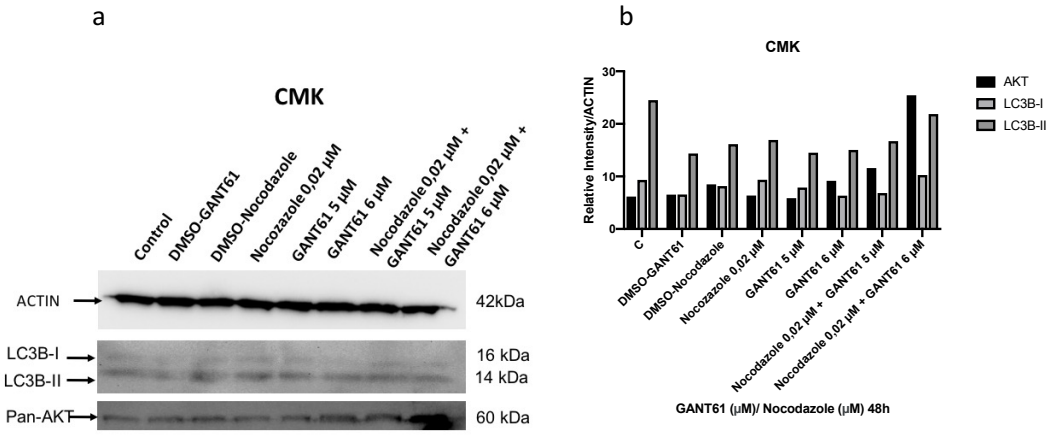

Figure 3.5.10 (a) The effect of GANT61/Nocodazole combination treatment the expression level of AKT and LC3B proteins in CMK cells for 48h (b) Graphical presentation shows the relative intensity of AKT and LC3B after normalization with Actin.

As a conclusion, after treatment with Ammonium Chloride, CQ and HCQ for 48h we observed the accumulation of LC3B-II in MOLM-13 and CMK cell lines. In addition, Nocodazole treatment led to a decrease in LC3B-II in MOLM-13 cells. Also, GANT61 and Nocodazole combination treatments led to the accumulation of LC3B in both MOLM-13 and CMK cells. Moreover, AKT protein expression changed depending on the type of treatment and cell lines. Based on our results, we can suggest that Hh pathway crosstalk with autophagy through mechanisms that needs further investigation.

Chapter 4

4. Conclusion and Future Prospects

4.1 Conclusion

Chromosomal abnormalities and aberrant signaling pathways has a major role in the pathogenesis of AML [6]. Many different studies reported that the Hedgehog signaling pathway is deregulated in AML. Hh pathway interacts with other pathways and regulates autophagy. Understanding the relationship between autophagy and Hh pathway could help to overcome the leukemic growth. Here we show the relationship between Hh pathway and autophagy in the MOLM-13 and CMK cell line *in vitro*. We used different autophagy modulators and Hh pathway inhibitor GANT61. Our data indicates that the combination of Hh pathway inhibitor GANT61 with autophagy inhibitors exhibited a synergistic effect on MOLM-13. However, the Hh pathway inhibitor GANT61 and autophagy inhibitors combination therapy resulted in antagonistic effect on CMK cell line. This suggests that combination treatment is a promising therapy for MOLM-13 AML cells but not CMK cells.

PP242, which is an ATP‑competitive inhibitor of mTOR kinase, causes induction of autophagy since mTOR is a negative regulator of autophagy [247-249]. We treated the cells with PP242 for activation of autophagy and we checked the anti-proliferative effect of PP242 on the CMK and MOLM-13 cells. We found that the proliferation of MOLM-13 cells (Figure 3.1.1.a) increased compared to DMSO control at the end of the 48h treatment period at the highest concentration (0.05 μM). In addition, the proliferation of CMK cells statistically didn't change at the highest concentration of PP242 (0.05 μM) compared to control (Figure 3.1.1.b). Our data contradicts many studies that have shown the anti-proliferative effect of PP242 on the different types of tumor cells. Xing et al. have shown that PP242 caused a decrease in the proliferation of gastric cancer cells. [267]. Another study showed that PP242 caused suppression of bladder

cancer cell proliferation [268]. Ono et al. reported that PP242 suppressed leukemic cell growth [269]. Moreover, Gordeev et al. demonstrated that the proliferation of tumor rodent E1A + cHa-Ras (ERas) cells significantly decreased after treatment with PP242 [270]. Moreover, the induction of autophagy via inhibition of mTOR by using PP242 reduced the cell proliferation of glioblastoma stem cells (GSCs) [271]. Depending on our results, we can suggest that chemical activation of autophagy have no significant effect on the survival of these cells. This is consistent with many studies that have suggested autophagy as a prosurvival mechanism [19-20]. However, in order to further prove this role further studies are needed. In addition, we have checked the role of the PP242 on AKT, a negative regulator of autophagy. The expression level of AKT protein decreased compared to DMSO control in the MOLM-13 cell line upon PP242 treatment. Consistent with our data, Morris et al. showed that AKT expression diminished in the HEK293 cells after treatment with PP242 [227]. To further understand effect of PP242 treatment on the molecular mechanisms of autophagy, we are planning to check the expression of LC3B, ATG5 and ULK1 proteins expression. Furthermore, to understand the relationship between the hedgehog and mTOR pathways and their role in the regulation of autophagy, we are planning to perform combination treatment experiment with Hh pathway inhibitor GANT61 and mTOR inhibitor PP242 on our AML cells and check the molecular markers of autophagy.

For autophagy manipulation, we chose different autophagy inhibitors such as Ammonium Chloride, Chloroquine, Hydroxychloroquine and Nocodazole based on guidelines that were published in Cell by Mizushima et al. [260]. We treated our cells with autophagy inhibitors and we demonstrated the antiproliferative effect of all inhibitors on both MOLM-13 and CMK (Figure 3.1.2, Figure 3.1.3, Figure 3.1.4 and Figure 3.1.5). Similarly, Baquier et al. reported the antiproliferative effect of Nocodazole on the proliferation of both myeloid leukemia cells and liver cancer cells [272]. Another study has shown the inhibitory effect of Chloroquine and Hydroxychloroquine on the growth of bladder cancer cells [262]. This was also detected by Fan et al. in lung cancer cells, where the proliferation of these cells decreased after CQ treatment [263]. In addition, Hu et al showed that treatment with CQ causes the suppression of the growth of liver cancer cells [264]. Our results were consistent with previous works and led to a decrease in the proliferation of AML cells.

GANT61, which is the first GLI antagonist, inhibits GLI1 and GLI2 transcription factor. For our study, we chose the GANT61 as a Hh pathway inhibitor. Before starting combination therapy, we conducted a single treatment of GANT61 on MOLM-13 and CMK cell line. Our study showed that the proliferation of both MOLM-13 and CMK cells decreased compared to the control in both a time-dependent and dose-dependent manner. Consistent with our study, Benvenuto et al. reported the antiproliferative effect of GANT61 on the breast cancer cell both in vivo and in vitro [273]. Another study showed that the treatment of the pancreatic cancer stem cells with GANT61 leads to the suppression of cell growth [274]. Hh inhibition with GANT61 decreased the proliferation of prostate cancer cells. In addition, in this study, they have shown that GANT61 was effective compared to SMO inhibitor GDC-0449 on the proliferation of prostate cancer cells [275]. Moreover, Pan et al. reported that the inhibitory effect of GANT61 on the different myeloid leukemia cells. In addition, they reported a synergistic effect of GANT61 and rapamycin on these cells [259]. All together, we can suggest that our data confirms the literature in that GANT61 treatment led to a decrease in cell viability of MOLM-13 and CMK.

In order to understand if the decrease in AML cell proliferation upon autophagy inhibition is due an increase in cell death, we treated the cells autophagy inhibitors for 48h and checked the apoptotic and necrotic cell populations using Annexin V staining, which was detected using flow cytometer. After ammonium chloride treatment, although the percentage of apoptotic cells didn't significantly change in MOLM-13 cells, the percentage of necrotic cells increased approximately 4 folds compared to water control in MOLM-13 cell line. (Figure 3.4.1.a). On the other hand, the apoptotic and necrotic cell percentage of CMK cell didn't change compared to water control after Ammonium Chloride administration for 48h (Figure 3.4.2.a). After that, we performed CQ treatment to check the cell death for both cells. After we treated the MOLM-13 cells for 48h, both the percentage of the apoptotic cells and necrotic cells increased at the IC80 value compared to the water control for MOLM-13 (Figure 3.4.1.b). Consistent with this result, Lin et al. reported that CQ and HCQ treatment promoted the apoptosis in the bladder cancer cells [262]. The other study shown that CQ leads to the induction of apoptosis in the lung cancer cells [263]. However, the apoptotic and necrotic cell percentage of CMK cells didn't change both at IC50 and IC80 values after treatment with CQ for 48h (Figure 3.4.2.b). When the MOLM-13 cells were treated with Hydroxychloroquine, we observed

that there is no change at the apoptotic cell percentage compared to the control (Figure 3.4.1.c). To better understand the role of our autophagy inhibitors on cell death in our cells, we need to perform further studies such as cell cycle analysis, detection of caspase 3/7 activities, checking the expression of cleaved caspase-3, Bax/Bcl-2 and poly(ADP-ribose) polymerase (PARP) cleavage by using immunoblotting.

Due to the importantance of both hedgehog pathway and autophagy in the leukemogeneis of AML, we wanted to check the effect of combination treatment of both GANT61 and autophagy inhibitors on AML cells in the hope that we can reveal a new therapeutic approach to prevent leukemic growth. With this aim, we performed combination of GAN61 and different autophagy inhibitors. After we determined the IC20 and IC30 values from the proliferation curves, we started the combination experiments. We performed GANT61+CQ and GANT61+Nocodazole combination treatments for MOLM-13. At the end of the 48h treatments, we concluded that both combination therapies led to a decrease in MOLM-13 cell viability compared to single treatments and DMSO-control (GANT61). Similarly, Wang et al. shown that combination treatment with Hh pathway inhibitor GANT61 and autophagy inhibitor 3-MA decreased the cell viability compared to single treatments in neuroblastoma cells [226]. Another study revealed that combination treatment of chloroquine and GANT61 led to increasing cytotoxicity compared to single treatments in hepatic stellate cells. [227]. On the other hand, the combination treatments in the CMK cell line differed compared to MOLM-13. We performed GANT61+NH_4CI and GANT61+Nocodazole combination treatments for the CMK cell line. GANT61+NH_4CI combination treatments resulted in an increase in cell viability of CMK cells compared to single treatments. For GANT61+Nocodazole combination, we observed that proliferation of CMK cells decreased compared with DMSO control and Nocodazole alone (0.03 μM), despite the fact that proliferation of CMK cells increased compared Nocodazole alone (0.03 μM) and GANT61 alone (5 μM and 6 μM). One explanation could be due to the different genetic background of CMK MOLM-13 cell line. MOLM-13 is an FLT3 mutated cell line while CMK is DS- AMKL. Moreover, many studies have shown that GANT61 promotes autophagy. Xu et al. showed that inhibition of Hh pathway with GANT61 lead to induction of autophagic pathways in pancreatic ductal adenocarcinoma cells [228]. In the case of combination treatments with autophagy and Hh pathway inhibitor, GANT61 may have triggered autophagy in CMK cell line, which have a low basal level of

autophagy[236]. These results confirm the prosurvival mechanism of autophagy on AML cells but further studies are required to understand which mechanisms are responsible for the different response to combination therapy.

Lastly, we checked the effect of our drugs and combinations treatments on the protein expression of LC3B which is an autophagy marker and AKT which is a negative regulator of autophagy. When we treat the cells with lysosomotropic autophagy inhibitors like NH4CI, CQ or HCQ we expected the accumulation of LC3B-II due to inhibition of autophagosome-lysosome fusion [253]. With this aim, we treated the MOLM-13 cells with Ammonium Chloride, CQ and HCQ for 48h in order to check LC3B-II accumulation. We observed that all inhibitors led to the accumulation of LC3B-II (Figure 3.5.1, Figure 3.5.2). Similarly, when we treated the CMK cells with lysosomotropic autophagy inhibitors NH4CI, CQ and HCQ, we observed the accumulation of LC3B-II (Figure 3.5.5, Figure 3.5.6) Consistent with our results, Mo et al. reported that the accumulation of LC3B-II increased after treatment with CQ compared to untreated cells on the retinal pigment epithelial (RPE) cells [276]. Moreover, Redmann et al. revealed that Chloroquine led to an increase in the level of LC3B-II protein expression in a dose-dependent manner [277]. This accumulation of LC3B-II as an autophagic marker after treatment with lysosomotropic autophagy inhibitors indicates a successful inhibition of autophagy. In addition to single treatments, we observed that the GANT61+NH_4CI combination treatment resulted in decreasing of LC3B-II in the CMK cells (Figure 3.5.10). Densitometric comparison of LC3B-II expression after the GANT61+Nocodazole combination treatment demonstrated a decreased LC3B-II expression compared to DMSO-control (GANT61) in CMK cell line (Figure 3.5.10). In our study, the use of GANT61 with NH_4CI may have caused induction of autophagy and degradation of LC3B-II. Similar to our results, Zeng et al. revealed that vismodegib, which is a Hh pathway inhibitor, induced autophagy and triggered lysosomal degradation of LC3B-II in the BCR-ABL positive chronic myeloid leukemia cells [278]. On the other hand, GANT61+Nocodazole combination therapy resulted in the accumulation of LC3B compared to DMSO-control (GANT61) on the MOLM-13 cells (Figure 3.5.8). This difference could be due to the low basal autophagy level in CMK cells as shown in a study done by Stankov et al [236].

In addition to LC3B, we checked the expression of AKT protein as negative regulator of autophagy after single treatments and combinational treatments. AKT

expression was changed based on the type of cells, drugs and the inhibition of the different steps of autophagy. Both GANT61+CQ and GANT61+Nocodazole combination therapy resulted in increased AKT expression compared to DMSO-control (GANT61) in MOLM-13 cells. While GANT61+NH_4CI treatment resulted in a decrease in the protein expression level of AKT, GANT61+Nocodazole increased the protein expression level of AKT compared to DMSO-control (GANT61) in CMK cell line. Milla et al. have shown that Hh directly regulates autophagy via the existence of multiple Gli consensus binding sites on the human ATG5 promoter [225]. In order further understand relationship between the Hh pathway and autophagy inhibition on AML cells, we are planning to check other autophagy related markers such as ATG5, p62, and ULK1 and importantly check the protein expression level of phosphorelated AKT, which is the active form of AKT.

In conclusion, our study have shown that autophagy and Hh pathway combination therapy exhibited a synergistic effect on MOLM-13 cells compared to single treatments. On the other hand, autophagy and Hh pathway combination treatments resulted in antagonistic effect in CMK cell lines compared to single treatment of GANT61. Furthermore, the GANT61/Chloroquine combination treatment resulted in accumulation of LC3B on MOLM-13 cells. Although the combination of GANT61 with NH4CI led to decreasing of both LC3B and AKT, GANT61/Nocodazole combination treatment resulted in accumulation of LC3B and AKT on the CMK cell line. Here we revelaled the crosstalk between autophagy and Hh pathway and its role on different AML cell lines. Also, we have demonstrated that the combination treatment of Hh and autophagy inhibitors is a promising therapy for AML with FLT3 mutations compared to AML with a DS-AMKL background.

4.2 Future Prospects

AML is the most common form of acute leukemia and it has a poor prognosis. According to data of the American Cancer Society, 21,450 new AML patients have been estimated in the United States and about 10,920 of these cases are expected to be fatal in 2019 [5]. Aberrant signaling pathways could drive the pathogenesis of AML. The Hedgehog signaling pathway is one of these pathways and it has a major role in regulation of autophagy. Several studies have shown that inhibition of the Hh pathway could decrease the chemoresistance and may overcome leukemic growth. Sadarangani et

al. reported that inhibition of GLI transcription factor abolished the leukemic proliferation [207]. In addition, Folkerts et al. demonstrated that hydroxychloroquine (HCQ) treatment result in prevention of leukemic growth in AML cells [238]. Thus, targeting these two pathways is important to understand the underlying mechanism of AML pathogenesis. In order to come up with a more targeted therapy based on Hh activation and the basal autophagy level of AML, we conducted combination therapies using the Hh pathway and autophagy inhibitors on AML cell lines. Autophagy and Hh pathway combination therapy exhibited synergistic effect on MOLM-13 cells compared to single treatments and resulted in decreasing of cell viability. To further confirm the synergistic effect of combination treatment that we have seen *in vitro* for MOLM-13, we will perform *in vivo* experiments on a xenotransplant mouse model of FLT-3 AML cells and patients.

On the other hand, the proliferation of CMK cells increased compared to single treatments and autophagy and Hh pathway combination treatments resulted in an antagonistic effect on CMK cell line. Our combination therapy did not show the expected effect for CMK cells. However, these results may be useful in understanding the mechanisms underlying abnormal signaling pathways in different AML cells. Consistent with literature, we suggest that Hh pathway inhibition induces autophagy for CMK cell line. This reveals that the pro-survival effect of autophagy in cancer cells. In order to understand this mechanism, we are planning to check gene expression level of Hh and autophagy related genes using PCR after combination experiments. Also, CMK and MOLM-13 have different background. While MOLM-13 is AML M5 cell line with an FLT3-ITD mutation, CMK is AML M7 cell line from down-syndrome patients. These study will help in understanding the underlying molecular mechanisms that leads to an aberrant Hh signaling pathway and dysregulated autophagy in different AML cell lines.

In addition, we checked the expression of autophagic marker LC3B and AKT expression after combination treatment. Densitometric analysis of of LC3B-II expression has shown the accumulation of LC3B-II in the both MOLM-13 and CMK cell line after GANT61+Nocodazole combination therapy. On the other hand, we revealed the expression level of AKT either decrease or increase depending on combination of Hh pathway inhibitor GANT61 with different autophagy inhibitors. In order better understand relationship between the Hh pathway and its role on autophagy inhibition on AML cells, we are planning to check expression level of other autophagy related markers such as ATG5, p62, and ULK1. Besides, we will check the phosphorylated AKT (p-AKT)

after combination treatments, which will give us a further insight on the molecular mechanisms that governs the pathogenesis of different subsets of AML.

Moreover, to clarify underlying mechanism of our autophagy inhibitors on cell death for AML cells, we need to perform further studies such as cell cycle analysis, detection of caspase 3/7 activities, checking the expression of cleaved caspase-3, Bax/Bcl-2 and PARP cleavage by using western blotting in the future.

In this study, we have shown the relationship between the Hh pathway and autophagy in the MOLM-13 and CMK cell line as *in vitro*. Also we have found that targeting both Hh pathway and autophagy is a promising therapy for AML with FLT3 mutations. As a conclusion, understanding the role of the hedgehog pathway on autophagy will help us design a new targeted personalized AML therapy based on the Hh expression and the basal autophagy level of AML cells that differs in the different subset of AML cell lines and patients.

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