AMERICAN UNIVERSITY OF BEIRUT

TARGETED THERAPY FOR ACUTE MYELOID LEUKEMIA: RETINOIC ACID, ACTINOMYCIN D, AND EVEROLIMUS

by
PERLA GEORGES MAHKOUL

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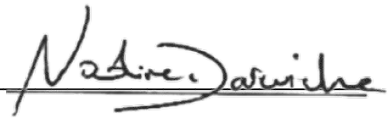

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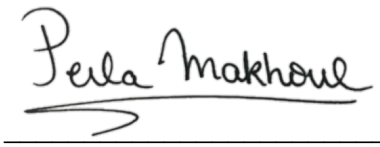

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ABSTRACT
OF THE THESIS OF

Perla Georges Makhoul for Master of Science
Major: Microbiology and Immunology

Title: Targeted Therapy for Acute Myeloid Leukemia: Retinoic Acid, Actinomycin D, and Everolimus

Acute Myeloid Leukemia (AML) is a heterogeneous malignancy of myeloid origin and one of the most common adult leukemias. Despite all therapeutic advances, AML still associates with poor prognosis, high relapse rates, and resistance to chemotherapy. Personalized medicine based on predicted response or risk of disease, is gaining major interest in AML management. This includes targeted therapies of specific mutations or disrupted pathways in AML. Everolimus (EV), an mTOR inhibitor, improved the treatment of AML patients when combined with other therapeutic agents. Actinomycin D (ActD), an anti-tumor antibiotic, inhibited AML cell growth *in vitro*, yet presents an unambiguous clinical efficacy in some relapsed/refractory AMLs. Retinoic acid (RA), a hormone playing a major role in differentiation, proved beneficial, alone or combined to other agents on some subtypes of AML.

In this study, we explored the effect of simultaneous administration of RA, EV, ActD or the sequential administration of RA first, followed by EV and/or ActD on AML. *In vitro*, we used OCI-AML2, OCI-AML3, THP-1 and MOLM-13, presenting different mutations and reflecting some heterogeneous groups of AML. *In vivo*, xenograft mice were injected with OCI-AML2 or OCI-AML3 and treated with either RA followed by EV and/or ActD, or simultaneously with RA/Act/EV. Mice were monitored for survival, or humanely sacrificed 2 weeks post-treatment. Liver weight was recorded, and leukemic burden was assessed by immunophenotyping.

We demonstrated that OCI-AML2, OCI-AML3, and MOLM-13 are sensitive to ActD alone or to EV/ActD combination and RA addition to these treatments did not confer any additional advantage in OCI-AML2 and OCI-AML3. THP-1 cells lacking p53, were less sensitive to ActD alone, yet the addition of EV to ActD resulted in a more pronounced cell death. This phenotype which remained less prominent than that observed in cells with intact p53. Adding RA to EV demonstrated some beneficial effect in all tested cells, but this effect was not as prominent as ActD or EV/ActD. Accordingly, we restricted our molecular analysis to EV/ActD. We revealed a p53-dependent apoptosis in OCI-AML2 and OCI-AML3, 48 h post-treatment. Similar to *in vitro* results, sequential treatment with RA first, followed by EV/ActD did not yield any beneficial *in vivo* antitumor effect. Indeed, mice presented with sustained hepatomegaly and high even more exacerbated tumor burden in bone marrow for some conditions. However, our preliminary results showed that EV single agent, or EV/RA/ActD for 5 days following initial administration of RA prolonged survival of OCI-AML3 xenografted mice, while EV and ActD single agents, and RA prior to EV/ActD prolonged survival of OCI-AML2 mice. In conclusion, our preliminary study

demonstrated that an original treatment with RA before EV and/or ActD may not be of great beneficial efficacy and is dependent on the AML subtype, but these results require a confirmation.

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ABBREVIATIONS

A	
Act D	Actinomycin D
AML	Acute Myeloid Leukemia
AN V	Annexin V
APL	Acute Promyelocytic Leukemia
ATRA	All-Trans Retinoic Acid
ATO	Arsenic Trioxide
ASXL1	Additional sex comb-like 1
B	
BCL2	Biosafety cabinet level 2
BM	Bone marrow
C	
CR	Complete remission
Cytarabine	Ara-C
E	
eIF4E	Eukaryotic initiation factor 4E
4E-BP1	Eukaryotic initiation factor 4E binding protein
ELN	European LeukemiaNet
EV	Everolimus
F	
FAB	French American British
FDA	Food and drug administration
FLT3	Fms-like tyrosine kinase 3
H	
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HMT	Histone methyltransferase
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplant
HDM2	Human counterpart of MDM2
I	
ITD	Internal tandem duplication
IDH	Isocitrate Dehydrogenase
L	
LSC	Leukemic stem cell
M	
MDM2	Murine double minute 2

mTOR	Mammalian target of rapamycin
mTORC	mTOR complex
N	
N-CoR	Nucleal receptor corepressor
NPM1	Nucleophosmin-1
wt-NPM1	Wild type NPM1
NPM1-c	Mutant NPM1
O	
OS	Overall Survival
P	
PI3K	Phospholnositide 3-Kinase
Pp53	Phospho-P53
PML	Promyelocytic leukemia
PML-NB	PML nuclear bodies
PtdSer	Phosphatidylserine
R	
Raptor	Refulatory-associated protein of Mtor
RA	Retinoic acid
RAR	Retinoic Acid Receptor
RARA	Retinoic Acid Receptor α
RXR	Retinoid X Receptor
S6K	Ribosomal protein S6 kinase
RUNX1	Runt-related transcription factor
S	
SMRT	Silencing mediator for retinoid and thyroid hormone receptor
SRC	SRB mediator-containing complex
T	
TP53	Tumor protein p53
W	
WHO	World Health Organization

CHAPTER I

ACUTE MYELOID LEUKEMIA

A. Pathophysiology

Acute myeloid leukemia (AML) is an aggressive heterogenous hematological malignancy [1]. AML starts in the bone marrow (BM), and results in an abnormal increased proliferation of progenitor cells and their inability to differentiate into mature cells (Figure 1). Consequently, a decrease in the production of normal mature blood cells and an accumulation of immature myeloid precursors lead to BM failure [2]. Poorly differentiated myeloblasts can move into the peripheral blood and may also spread to other organs such as the spleen, liver, or lymph nodes, where they interfere with their normal functions [3].

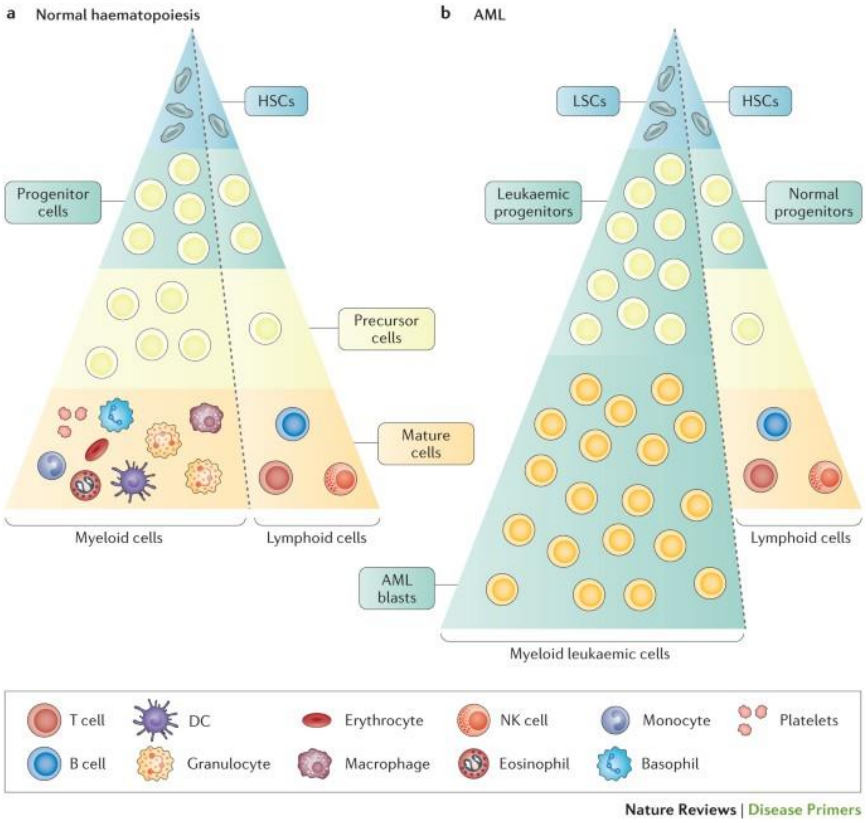


Figure 1. Normal and leukemic hematopoiesis [4]

a. General hierarchical structure of normal hematopoiesis: hematopoietic stem cells (HSCS) give rise to progenitor cells, which produce precursor cells and finally mature blood cells as indicated. **b.** Aberrant hematopoiesis in AML: leukemic stem cells (LSCs) give rise to AML progenitor cells and the more mature but morphologically primitive myeloblasts that make up the bulk of the neoplasm.

B. Epidemiology

AML is one of the most common types of adult leukemia, accounting for around 80% of acute leukemias [5], and around 30% of total leukemia cases [6]. Of all leukemic subtypes, AML accounts for the highest percent of deaths (62%) [7]. AML can occur at any age group and classifies into pediatric AML and adult AML. In adults, the median age is around 68 and the incidence sharply increases with age [8]. Patients older than 60 have a poor prognosis [9], with an overall survival (OS) of less than 30% at 5 years [10].

C. Etiology

In most patients, AML develops spontaneously without any predisposing condition or antecedent disease. It appears in healthy individuals as a result of the acquisition of mutations at the level of hematopoietic stem cells (HSCs) or myeloid progenitor cells [11], leading to the development of the disease [12]. But it can also arise as a consequence of environmental exposures, prior to hematological malignancies or therapies [13].

D. Clinical presentation

Clinical manifestations of AML result from the clonal expansion of undifferentiated malignant myeloblasts, which interferes with normal hematopoiesis. As a result, AML patients display signs of thrombocytopenia, anemia, and neutropenia

[14]. Other common symptoms among patients include fatigue, shortness of breath, weakness, dizziness, fever, excessive bruising, and bleeding [15, 16]. In addition, AML patients experience an increased risk of infections due to the prevalence of neutropenia [17]. Other clinical features include lymphadenopathy, splenomegaly, hepatomegaly [3], hemorrhage, and gingival bleeding [18], and the most serious, albeit rare complications, are intracranial bleeding (5%) [19] and choloroma (otherwise known as granulocytic/myeloid sarcoma) (2-9%) [20].

E. Recurrent mutations

Chromosomal deletions or translocations are reported in 50% of AML cases [21], while the remaining 50% of patients exhibit normal cytogenetics with gene mutations [22]. Whole genome sequencing of 200 AML patients by the Cancer Genome Atlas Research Network allowed the identification of eight functional gene categories that are commonly mutated in AML (Figure 2). These include signaling genes like the *tyrosine kinase receptor gene FMS-Like tyrosine kinase 3* (*FLT3)*, genes involved in DNA methylation such as *Isocitrate dehydrogenase* (*IDH1* and *IDH2)*, tumor suppressor genes such as *p53*, *Nucleophosmin-1 gene* (*NPM1)*, as well as many other genes involved in hematopoietic differentiation and transcriptional regulation such as the Runt-related transcription factor (*RUNX1*), additional sex-comb-like 1 (*ASXL1*) responsible for epigenetic cell homeostasis, and spliceosome-complex genes [23, 24].

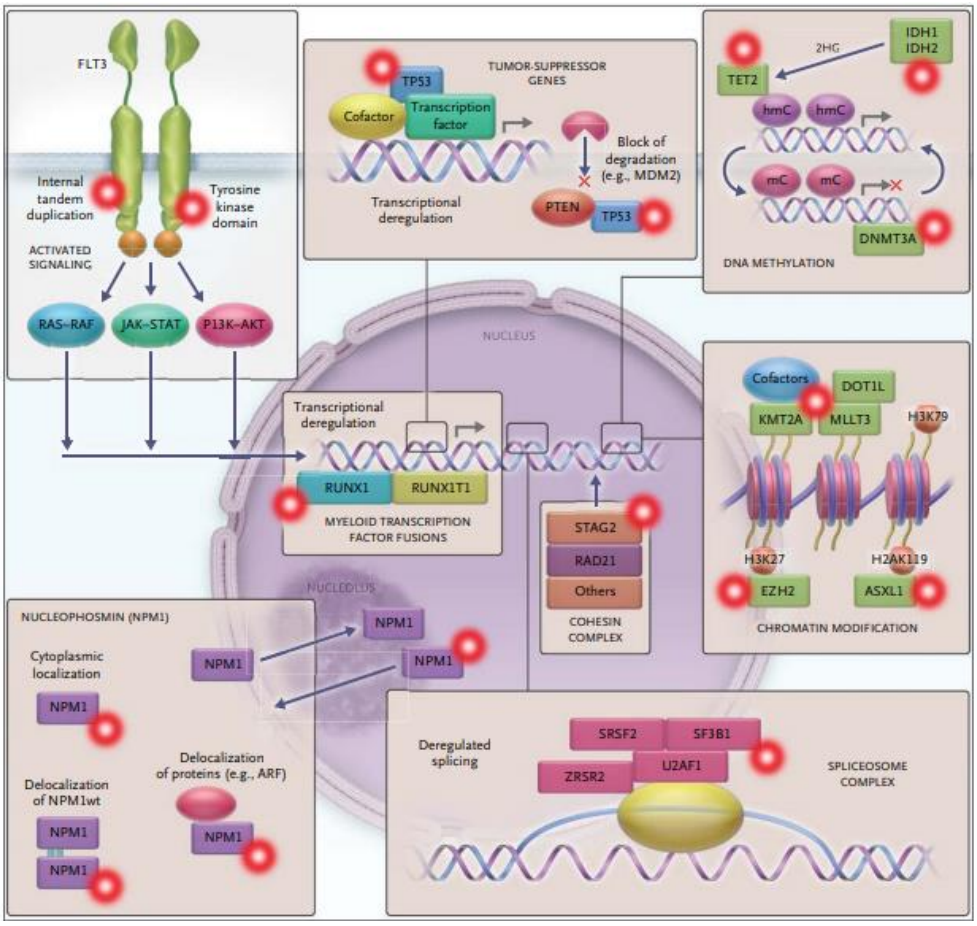
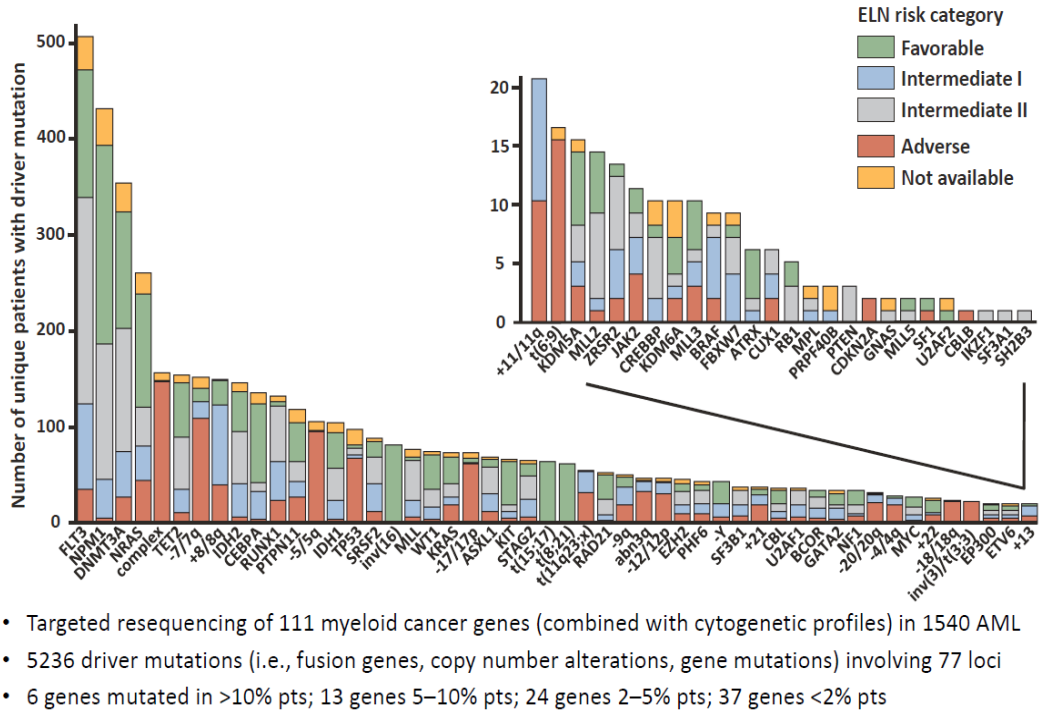


Figure 2. Eight functional categories of genes that are commonly mutated in Acute Myeloid Leukemia [23].
Mutations in signaling genes (*FLT3*) confer a proliferative advantage through RAS-RAF, JAK-STAT and PI3K-AKT signaling pathways (upper left box). Mutations in myeloid transcription factors (*RUNX1*) lead to transcriptional deregulation and impaired differentiation (center left box). Mutations in *NPM1* gene result in aberrant cytoplasmic localization of NPM1 (lower left box). Mutations of spliceosome-complex genes are involved in deregulated RNA processing (lower right box). Cohesion-complex gene mutations impair chromosomal segregation and transcriptional regulation (center middle box). Mutations of genes involved in epigenetic homeostasis (*ASXL1* and *EZH2*) lead to deregulation of chromatin modification (center right box). Deregulation of DNA methylation (*DNMT3A*, *TET2*, *IDH1* and *IDH2* mutations) (upper right box). Mutations in tumor suppressor genes (TP53) lead to transcriptional deregulation and impaired degradation (upper middle box).

Moreover, exome sequencing in AML patients confirmed most of aforementioned mutations and identified more than 20 driver recurrent mutations [25]. In addition to *NPM1*, *FLT-3*, *IDH*, *p53*, *RUNX1*, *ASXL-1* mutations, *DNA Methyltransferase 3A*

(*DNMT3A*) mutations, *Ten–Eleven Translocation 2* (*TET2*) mutations, *CCAAT Enhancer Binding Protein α* (*CEBPA*) mutations, *Mixed Lineage Leukemia* (*MLL*), *c-Kit* mutations, *Splicing Factor Gene* mutations, and Cohesion Complex Members mutations were also identified (Figure 3) [26].



- Targeted resequencing of 111 myeloid cancer genes (combined with cytogenetic profiles) in 1540 AML
- 5236 driver mutations (i.e., fusion genes, copy number alterations, gene mutations) involving 77 loci
- 6 genes mutated in >10% pts; 13 genes 5–10% pts; 24 genes 2–5% pts; 37 genes <2% pts

Figure 3. The driver mutations identified in AML [26].
Driver events in 1540 patients with AML. Each bar represents a distinct driver lesion; the lesions include gene mutations, chromosomal aneuploidies, fusion genes, and complex karyotypes. The colors in each bar indicate the molecular risk according to the European LeukemiaNet (ELN) classification.

F. Classification

AML is a complex and highly polyclonal disease that evolves over time. At diagnosis, AML can present with multiple clones and different mutations. The heterogeneity and complexity of AML resulted in several classifications, which were revised with the revolution of sequencing techniques. This progress in unraveling AML heterogeneity and determining the appropriate AML subtype, improved both the prognosis and the treatment of the disease. In that sense, for each patient, the AML

subtype is currently dictating the choice of the treatment regimen. Currently, three main classification methods for AML are adopted.

1. The French-American-British (FAB) classification

Established in the 1970s, the first classification of AML was conducted by the French-American British (FAB) system, and divided the disease, according to the morphology, maturity, origin, and immune-histochemical features of AML cells, into eight major subtypes (M0 to M7) (Table 1) [27].

Table 1. The French-American-British (FAB) classification of AML [27].

Fab subtype	Name	Origin
M0	Undifferentiated acute myeloblastic leukemia	Starts in immature forms of white blood cells (WBC).
M1	Acute myeloblastic leukemia with minimal maturation	
M2	Acute myeloblastic leukemia with maturation	
M3	Acute promyelocytic leukemia (APL)	
M4	Acute myelomonocytic leukemia	
M4 eos	Acute myelomonocytic leukemia with eosinophilia	
M5	Acute monoblastic/monocytic leukemia	
M6	Acute erythroid leukemia	Starts in very immature forms of red blood cells (RBC).
M7	Acute megakaryoblastic leukemia	Starts in immature forms of cells that make platelets.

2. The World Health Organization (WHO) classification

Advances in the discovery of AML genetic alterations, their relevance and implication at the clinical level, led to revise the previous FAB system. Thus, in an

effort to build a classification system that facilitates the clinical management of AML, the World Health Organization (WHO) released, in 2016, a revised version of AML classification. This version categorizes the disease into six distinct subtypes depending on the cytogenetic changes and immunophenotypes (Table 2) [28].

Table 2. WHO 2016 categories of AML [28, 29].

AML with recurrent genetic abnormalities
AML with t(8;21) (q22;q22); RUNX1-RUNX1T1
Acute promyelocytic leukemia with t(15;17) (q24.1;q21.2); PML-RARα
AML with inv(16) (p13.1q22) or t (16;16) (p13.1;q22); CBFβ-MYH11
AML with t(9;11) (p21.3;q23.3); KMT2A-MLLT3
AML with t(6;9) (p23;q34.1); DEK-NUP214
AML with inv(3)(q21.3q26.2) or t (3;3) (q21.3;q26.2); GATA2, MECOM
Acute megakaryoblastic leukemia with t (1;22) (p13.3;q13.1); RBM15-MKL1
AML with t (9;22) (q34.1;q11.2); BCR-ABL1
AML with *NPM1* mutation
AML with biallelic *CEBPA* mutation
AML with *RUNX1* mutation
AML with myelodysplasia-related changes
Therapy-related myeloid neoplasms
AML, not otherwise specified
AML with minimal differentiation
AML without maturation
AML with maturation
cute myelomonocytic leukemia
Acute monoblastic/monocytic leukemia
Acute erythroid leukemia
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferation associated with Down syndrome[2]
Transient abnormal myelopoiesis
Myeloid leukemia associated with Down syndrome[1,2]

[1] Encompasses myelodysplastic syndromes as well as AML.
[2] WHO terminology.

3. The European Leukemia Net (ELN) classification

The revolution in sequencing technologies and the discovery of new genetic mutations in AML, urged further revision in AML classification. Updated in 2017, the European Leukemia Net (ELN) classification subdivided AML patients into three categories (Table 3), correlating the different genetic abnormalities and molecular alterations with expected treatment response and outcomes [30]. Indeed, the favorable category of AML patients corresponds to genetic changes, which associate, with good response to therapy. The intermediate category corresponds patients with normal cytogenetics, but which associates with less favorable prognosis. Finally, the adverse category of AML manifests with chromosomal changes associated with a high-risk of treatment failure.

Table 3. 2017 ELN risk stratification by genetics [30]

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*** Mutated *TP53*

* Low, low allelic ration (<0.5); high, high allelic ration (>0.5)
** These markers should not be used as an adverse prognostic marker if they occur with favorable-risk AML subtypes.

G. Current treatment modalities of AML

Because of the genetic complexity and heterogeneity of AML, treatment strategies remain variable among patients. The standard treatment of AML remained unchanged for more than 30 years. Therapeutic approaches of AML were only implemented during the last decade, which witnessed advances in understanding the pathophysiology of the disease following the revolutionized advances in molecular and sequencing techniques. Accordingly, personalized therapies targeting key driving mutations are currently adopted in newly diagnosed or relapsing/refractory patients, while a lot of therapies are still under pre-clinical or clinical investigation [31].

In newly diagnosed AML patients, and according to the Leukemia and Lymphoma Society, current AML management relies on aggressive chemotherapy administered in two phases: an induction phase whose goal is to eliminate AML leukemic cells, and a consolidation phase whose goal is to prevent the relapse of the disease (Figure 4). Indeed, most adult AML patients eligible for standard chemotherapy are treated with cytarabine (Ara C) for 7 days, with a combination of daunorubicin or idarubicin the first 3 days [32]. As such, the induction phase is referred to as the “7+3” regimen. Nowadays, if the patient fulfils specific criteria, recent studies recommend additional drugs to the previous combination in an attempt to increase the effectiveness of the treatment and increase the long-term survival rate [34, 35]. For example, targeted monoclonal antibody Gemtuzumab Ozogamicin (GO) is approved for CD-33 positive

AML patients and added to the high-dose cytarabine-based chemotherapy (7 + 3 + GO) [9, 32, 36].

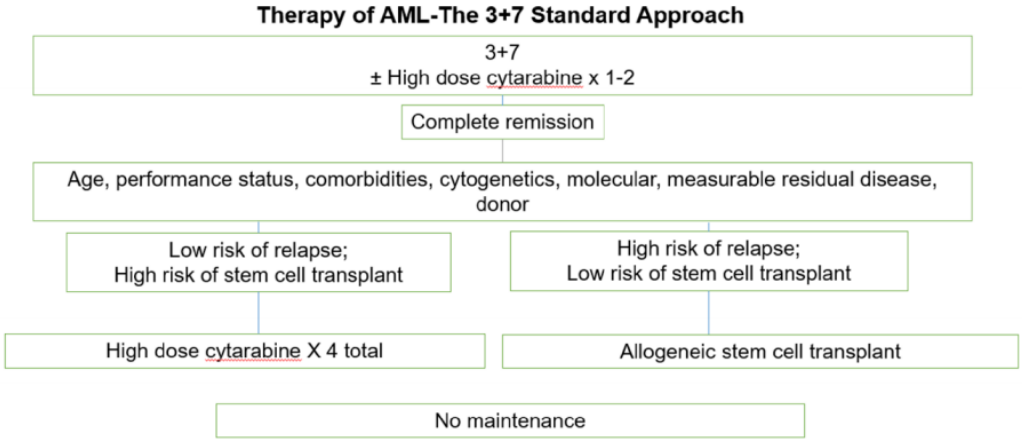


Figure 4. The 7+3 standard of care in AML [9]
Induction chemotherapy consists of treatment with anthracycline plus cytarabine-based regimen (3+7). After complete remission, the course of the treatment depends on patient's health status as indicated. Patients with low risk of relapse receive consolidation therapy consisting of high dose cytarabine (4 cycles). Patients with increased risk of relapse receive allogeneic hematopoietic stem cell transplant.

Patients who achieve complete remission (CR) from the induction therapy, are eligible for post-remission consolidation therapy [33]. This second phase is given in cycles with resting periods between each treatment, with the ultimate goal of eliminating any residual leukemia cells thereby preventing relapse [32]. It is also worth noting that risk assessment and eligibility are critical during the post-remission phase. Indeed, treatment is determined following the ELN guidelines [30] and dependent on the patient's fitness, age, performance status and comorbidities [9]. Patients with favorable risk outcomes are given intermediate dose cytarabine, while those with intermediate or adverse risk are treated with chemotherapy in addition to allogeneic hematopoietic stem cell transplantation (HSCT) [32]. Moreover, patients with increased risk of relapse in the first remission also receive HSCT.

H. Relapse and resistance

Despite the relatively high rate of complete remission following chemotherapy, AML still associates with high relapse rate in 66% of patients. In most cases, relapse arises within the first five years post therapy, but late relapse may also occur after 5 years of remission [37-39]. This is due to chemoresistance or acquired mutations in residual leukemic cells following initial treatment (Figure 5) [40].

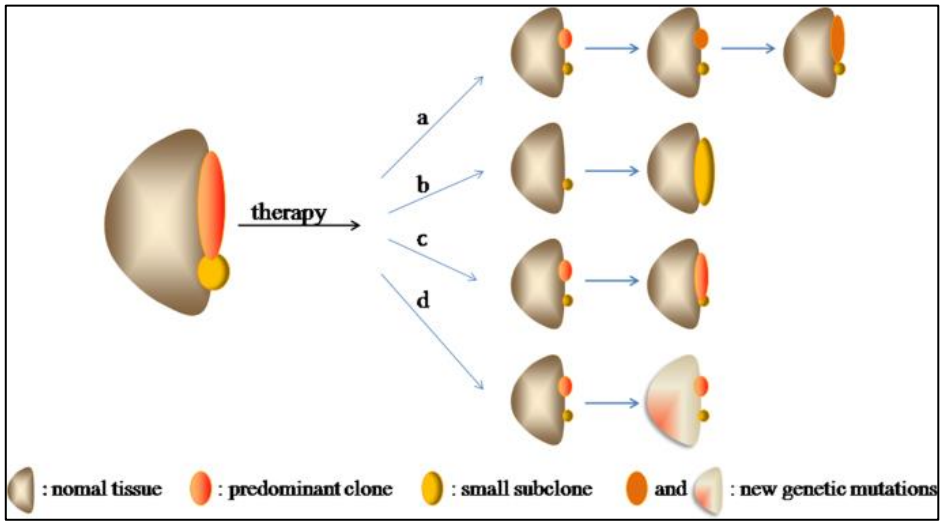


Figure 5. Pathogenesis of relapsed and refractory AML [41]
a. New mutations occurring on the basis of pre-existing mutation; **b.** small subclones evolving after the predominant clone was eradicated by therapy; **c.** relapse resulting from incomplete treatment; **d.** previously normal HSCs acquiring additional mutations and becoming malignant.

Treatment of relapsed AML patients is challenging and depends on their suitability for intensive chemotherapy and allogeneic HSCT. Fit patients receive standard cytarabine/anthracycline based induction chemotherapy, followed by HSCT after achieving CR, as this may induce long-term survival. Unfit relapsed AML patients to standard chemotherapy, receive either low dose of cytarabine or hypomethylating agents combined with venetoclax [32].

CHAPTER II

TARGETED THERAPIES FOR AML

Despite the availability of various chemotherapy regimens, AML management is still complicated due to the genetic heterogeneity of the disease. The outcome following chemotherapy, remains unfavorable due to relapse and resistance. In addition, a lot of elderly patients remain unfit to intensive chemotherapy and face different challenges ranging from increased incidence of comorbidities to higher mortality, and the "7+3" standard of care results in less than 15% survival rate [42, 43]. Reaching new frontiers in understanding AML pathophysiology at the cytogenetic and molecular led to the development of personalized therapeutic strategies designed according to specific mutations and molecular abnormalities. Since 2017, nine agents have been approved by the food and drug administration (FDA) [9]. These include combinations of epigenetic therapy with hypomethylating agents, addition of FLT3 inhibitors or venetoclax to chemotherapy, IDH inhibitors, p53 modulators, and menin inhibitors. Among the FLT3 inhibitors, it is worth noting that the first-generation inhibitor, sorafenib, exhibited a potent activity against AML [44, 45]. Giltertinib, a second-generation FLT3 inhibitor, was also FDA approved for the treatment of relapsing or refractory AML with a *FLT3* mutation [46]. In adult AML patients with complex karyotype but without *NPM1* mutation, induction therapy is not effective alone and should be complemented with hypomethylating agents such as decitabine and azacitidine [47-49]. Moreover, a meta-analysis of randomized studies demonstrated that Gemtuzumab Ozogamicin could be safely added to conventional induction therapy, and led to reduced relapse risk and a

significant survival benefit for patients with intermediate, and particularly favorable cytogenetics [34, 50].

Current targeted strategies also focus on establishing maintenance therapy, replacing parenteral therapies with oral drugs and using chimeric antigen receptor (CAR-T) therapy to boost the T lymphocyte mediated immune response (Table 4) [9].

Table 4. Recent FDA drug approvals (since 2017) in AML [9].

Treatment (approval date)	Description	Indication
Midostaurin (April 2017)	Multikinase FLT3 inhibitor	Newly diagnosed *FLT3*-mutated (as detected by FDA-approved test) AML, in combination with standard cytarabine and daunorubicin induction and cytarabine consolidation
Gemtuzumab ozogamycin (September 2017)	Anti-CD33 antibody–drug conjugate	Adults with newly diagnosed CD33-positive AML; refractory-relapsed CD33-positive AML in patients ≥ 2 years of age
CPX-351 (August 2017)	Liposomal cytarabine and daunorubicin at a fixed 5:1 molar ratio	Newly diagnosed therapy-related AML, secondary AML or AML with myelodysplasia-related changes
Glasdegib (November 2018)	Hedgehog pathway inhibitor	Newly diagnosed AML aged ≥ 75 years or with co-morbidities that preclude the use of intensive induction chemotherapy (in combination with low-dose cytarabine)
Venetoclax (November 2018)	BCL-2 inhibitor	In combination with azacitidine or decitabine, or low-dose cytarabine in newly diagnosed AML aged ≥ 75 years or with co-morbidities that preclude the use of intensive induction chemotherapy
Enasidenib (August 2017)	IDH2 inhibitor	Relapsed or refractory *IDH2*- mutated AML (as detected by FDA-approved test)
Ivosidenib (July 2018) (May 2019)	IDH1 inhibitor	1. Relapsed or refractory *IDH1*-mutated (susceptible mutation, as detected by FDA-approved test) AML. 2. First line treatment of *IDH1*-mutated AML (as detected by FDA-approved test), patients ≥ 75 years old or ineligible to receive intensive chemotherapy.
Gilteritinib (November 2018)	FLT3 inhibitor	Patients with relapsed or refractory *FLT3*-mutated AML (as detected by FDA-approved test)
CC-486 (September 2020)	Oral azacitidine hypomethylating agent (30% absorption) approved at 300 mg daily × 14 every month	Continued treatment of adult patients with AML who achieved first complete remission or complete remission with incomplete blood count recovery following intensive induction chemotherapy and who are not able to complete intensive curative therapy
Oral Decitabine-cedazuridine (July 2020)	Oral hypomethylating agent (100% absorption)	Alternative to parenteral HMAs decitabine for the treatment of adults with MDS (pretreated/untreated; de novo/secondary) or CMML

Other targeted therapies of AML may also include all-*trans* retinoic acid combination, everolimus (EV), and actinomycin D (ActD), which we will emphasize on throughout our study.

A. All-*trans* Retinoic Acid (ATRA)

1. An overview

Retinoids are signaling molecules that regulate cell differentiation and proliferation, as well as early embryonic development [51]. ATRA belongs to the family of retinoids, and is an active metabolite and a derivative of vitamin A. Prior research depicted ATRA as a promising regimen for treating multiple malignancies including hepatoma, lung cancer, breast cancer, and prostate cancer [52]. Most of the anti-tumor actions of ATRA are mediated through the activation of two nuclear receptors: Retinoid acid receptor (RAR) and Retinoid X receptor (RXR) [53]. In the absence of ATRA, RAR and RXR heterodimerize and localize into the nucleus, then bind to Retinoic Acid Response Elements (RARE) in the promoter region of their target genes. This recruits a corepressor protein, which inhibits transcription through chromatin condensation via histone deacetylation (Figure 6) [54, 55]. Upon binding of ATRA, conformational change in the ligand binding domain results in the dissociation of the corepressor complex, and recruitment of coactivator complex, resulting in chromatin de-condensation and subsequent transcriptional activation (Figure 6) [56]. This induces cell differentiation and cell cycle arrest resulting in the apoptosis of malignant cells [51].

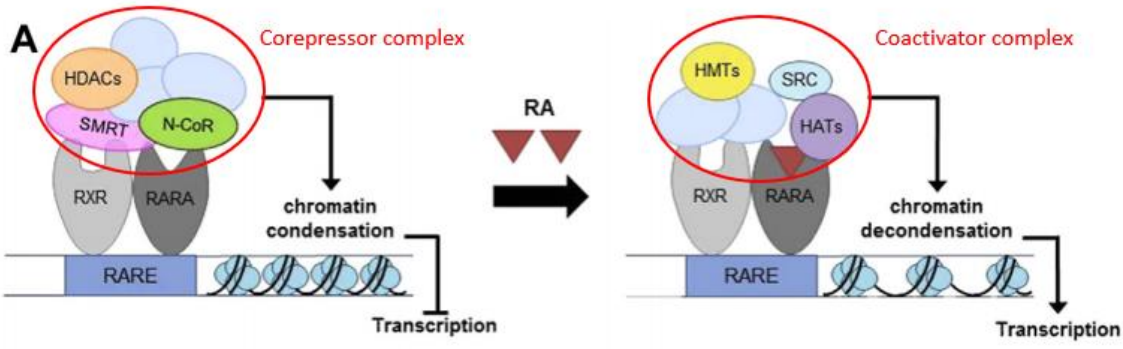


Figure 6. Mechanism of transcriptional regulation by RARs before and after biding of RA [57].
Heterodimers of RA and RXR bind to RARE in the promoter region of their genes. In the absence of ligand, corepressor complex is formed including N-CoR, silencing mediator for retinoid and thyroid hormone receptors (SMRT) and histone deacetylases (HDACs), which results in chromatin condensation. Upon RA binding, coactivator complex is recruited and includes histone methyltransferases (HMTs), histone acetyltransferases (HATs), and SRB mediator-containing complex (SRC), which leads to transcriptional activation of target genes through chromatin condensation.

2. ATRA in the treatment of Acute Promyelocytic Leukemia

Acute promyelocytic leukemia (APL) is a subtype of AML. According to the FAB classification, APL is an AML-M3 [27]. APL is characterized by a chromosomal translocation between the promyelocytic leukemia gene (PML) on chromosomes 15 and the retinoic acid receptor alpha (RARA) on chromosome 17, denoted as t(15;17) [58]. As a result, PML-RARA fusion protein is expressed, thereby blocking differentiation and apoptosis of progenitor cells, leading to leukemia [59].

ATRA induced differentiation of promyelocytic cells and led to major advances in APL treatment [60]. Indeed, ATRA combined with Arsenic Trioxide (ATO) induced degradation of PML-RARA fusion protein, and synergistically triggered APL regression [61]. ATRA/ATO combination marked remarkable cure rates in low/intermediate risk APL patients, thereby revolutionizing cancer treatment without the need for aggressive chemotherapy [62]. This combination is currently the golden standard treatment of APL. In that sense, low/intermediate risk APL patients exhibit OS rates ranging from 86% after three years to 99% after four years, following ATRA/ATO treatment [63-66].

3. ATRA in treatment of non-APL AML

In non-APL AML patients harboring the *NPM1* mutation, ATRA combined with other agents emerged as an efficient treatment [67]. *NPM1* gene encodes for a nucleolar phosphoprotein with continuous shuttling activity between the cytoplasm, the nucleus, and the nucleolus. Wild type NPM1 (wt-NPM1) protein is implicated in multiple cellular functions, including embryogenesis, centrosome duplication, ribosomal synthesis, and DNA repair [68]. When the gene is mutated, a cytoplasmic mutant protein (NPM1-c) is expressed, and exhibits an impaired ability to translocate into the nucleolus. This leads to the ectopic accumulation of the NPM1-c, oligomerizing with wt-NPM1 in the cytoplasm [69]. NPM1 mutations are frequent among AML patients, accounting for around 35% of AML adults [23], and they have been implicated in leukemogenesis and result in impaired differentiation and apoptosis in leukemic cells [67].

In *NPM1-c* AML, addition of ATRA to conventional chemotherapy remains controversial. While some studies showed that this regimen improves survival [70], others revealed that it did not [71, 72]. Preclinical studies investigated the combination of ATRA with other agents including ATO. In that sense, ATRA and ATO synergistically induced the proteasomal degradation of NPM1-c, the nucleolar re-localization of wt-NPM1 and led to the activation of the p53 pathway. This resulted in AML growth arrest and apoptosis in AML cells (Figure 7) [73]. Importantly, treatment of a few *NPM1-c* elderly AML patients with ATRA and ATO reduced bone marrow and peripheral blood blasts [73].

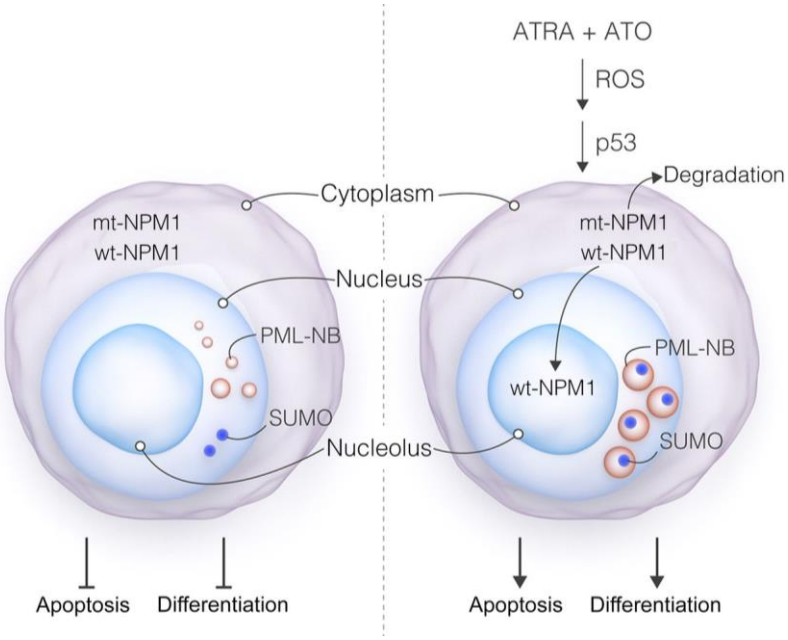


Figure 7. ATRA/ATO synergize to induce differentiation and apoptosis in NPM1-mutant AML cells [67].
Mutant NPM1 leads to delocalization of both mt-NPM1 and wt-NPM1 from the nucleolus to the cytoplasm, and results in disorganization of PML nuclear bodies (PML-NB) (small size, heterogeneous appearance, nucleoplasmic localization, and dissociation from SUMO-1). This disrupts cellular differentiation and apoptosis, resulting to leukemogenesis. ATRA/ATO triggers oxidative stress and p53 activation, culminating in mt-NPM1 degradation, re-localization of wt-NPM1 to the nucleolus, and reversal of PML-NB disorganization (larger size, more homogeneous appearance, and restoration of the SUMO-1 association). These events lead to leukemic cell differentiation and/or cell death.

B. Actinomycin D (Act D)

1. An overview

The tumor suppressor protein p53 plays a prominent role in apoptosis and cell cycle regulation [74] . In response to cellular stress, p53 induces apoptosis through two distinct but converging pathways: the intrinsic (or mitochondrial) pathway and the extrinsic (or death receptor) pathway [75]. In an attempt to survive and escape apoptosis, malignant cells impair p53 functions by activating its primary cellular inhibitor, HDM2, the human counterpart of murine double minute 2 (MDM2) in mice

[76]. This ubiquitin ligase targets p53 for proteasomal degradation [77]. Another mechanism by which malignant cells impair p53 functions, is by either inactivating the protein or inducing mutations in the *TP53* gene [78].

Actinomycin-D (ActD) is an antibiotic isolated from *Streptomyces* sp. with antitumor activities [79]. ActD is a DNA intercalator that forms a stable complex with GC residues of the DNA. This leads to the disruption of the RNA synthesis through RNA Polymerase I, resulting in the inhibition of ribosome biogenesis [80, 81]. At low concentrations, ActD decreases the activity of MDM2 thereby stabilizing and activating p53, explaining its beneficial therapeutic use in the treatment of cancer [82]. Of note, ActD can also induce growth inhibition in cells with deleted or mutated p53, which may also suggest a p53-independent mechanism of action [83].

2. ActD in the treatment of AML

In vitro studies in AML cell lines showed that low concentrations of ActD induce wt-NPM1 relocalization into the nucleoplasm, cell cycle arrest, and subsequent cell death [84]. Clinically, ActD treatment in AML patients harboring *NPM1* mutations and unfit to chemotherapy, yielded promising results [85, 86]. In that sense, an elderly AML patient with *NPM1-c* but without *FLT3-ITD* achieved complete remission (CR) after two cycles of ActD [85]. Furthermore, five days of treatment with ActD induced CR in two out of six patients, with relapsed/refractory *NPM1-c* AML [85]. A recent clinical trial showed that three patients out of seventeen with relapsed/refractory *NPM1-c* AML achieved CR following one cycle of therapy with ActD [86].

C. Everolimus (EV)

1. An overview

The phosphoinositide 3-kinase/Akt/mammalian target of rapamycin pathway (PI3K/Akt/mTOR pathway) modulates cell metabolism, proliferation, and survival [87]. In AML, this pathway is activated in about 60% of patients, leading to a notable decrease in OS and depicting it as a potential target for therapy [88, 89]. mTOR is a serine/threonine protein kinase, and its activity is accomplished by two complexes: mTOR complex 1 (mTORC 1) and mTOR complex 2 (mTORC 2). mTORC 1 is composed of mTOR, regulatory-associated protein of mTOR (Raptor), and 3 other proteins [90]. This complex is responsible for the phosphorylation of two downstream targets: the ribosomal protein S6 kinase (S6K) and the eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4E-BP1). S6K and 4E-BP1 are constitutively phosphorylated in AML, whereby S6K is activated and 4E-BP1 is inactivated, leading to enhanced protein translation and cell growth [91]. Everolimus (EV), a rapamycin analogue, is an mTORC 1 inhibitor exerting its actions by inducing cell cycle arrest and angiogenesis suppression (Figure 8) [92].

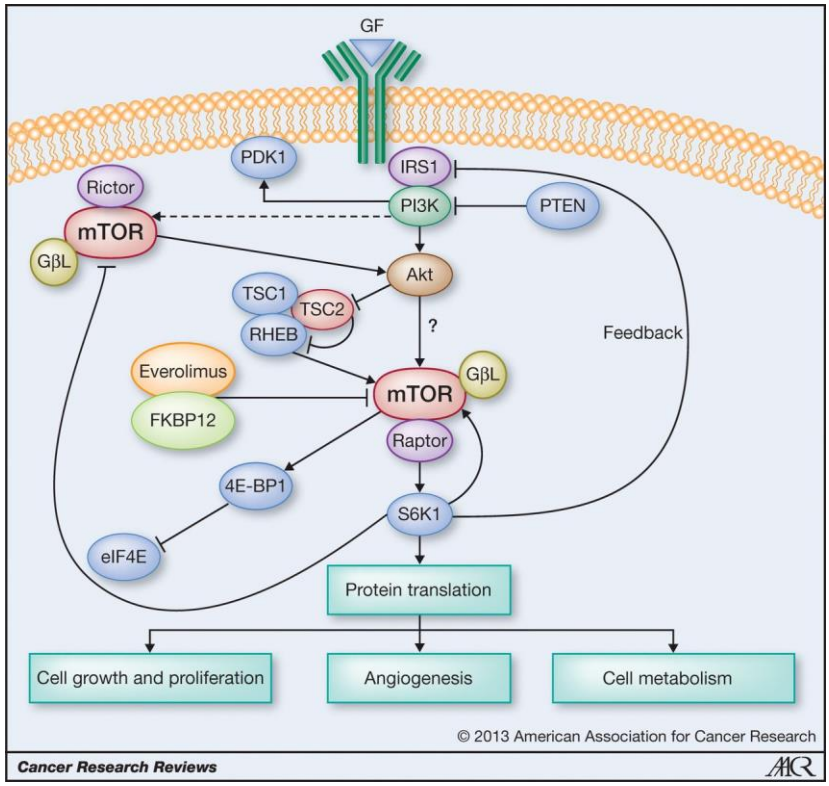


Figure 8. PI3K/Akt/mTOR pathway and EV mechanism of action [93].
Inhibition of mTOR by Everolimus may initiate a feedback loop mediated by S6K and IRS1 that results in the upregulation of Akt.

2. EV in the treatment of AML

EV was used for treating a panoply of solid tumors, including metastatic breast cancer [94, 95] and renal cell carcinoma [96, 97]. EV was also used in AML treatment, where several studies and clinical trials reported cell growth inhibition when it is combined with different drugs [98-101]. For example, when combined with the PI3K/Akt inhibitor IC87114, EV induced synergistic inhibition of blast cells proliferation [98]. Moreover, a clinical study on 28 AML patients in first relapse examined the effect of adding EV to conventional chemotherapy. When EV was administered at days 1 and 7 of the induction phase, 19/28 individuals (68%) achieved CR [99]. A recent clinical trial on 40 AML patients combined EV with Azacitidine, a nucleoside analogue that induces DNA hypomethylation to overcome the differentiation

block in AML [100]. This combination yielded promising clinical activity with an increased OS by 8.5 months [101].

D. ATRA and EV combination in treatment of APL

The combination of ATRA and EV was tested in APL. *In vitro*, ATRA/EV combination enhanced cell differentiation and growth arrest in HL60 and NB4 cells. Furthermore, ATRA augmented the EV-stimulated inhibition of mTORC1 signaling by upregulating negative regulators of mTORC1 [102]. *In vivo*, ATRA/EV reduced tumor weight and volume in mice, as compared to single agents [102]. This study highlights the promising use of ATRA/EV combination in APL, and warrants to evaluate its effect on non-APL AMLs.

CHAPTER III

AIM OF THE STUDY

AML is one of the most frequent and complex leukemias in adults. To date, and despite the advances of DNA sequencing techniques and the revolutionized personalized medicine, a lot of AML subtypes still represent an unmet medical condition, with either poor prognosis and high rates of relapse and resistance to standard chemotherapy, hence requiring more appropriate therapeutic regimens. In light of the absence of a golden standard treatment for AML, we investigated the effect of clinically approved drugs, namely retinoic acid (RA), EV and ActD in AML therapy. Indeed, RA proved effective, when combined with ATO in APL, while its efficacy in non-APL AML remained controversial. ActD proved efficient in certain *NPM1-c* AML especially in relapse refractory cases, yet the number of patients remained limited, and the studies were not conclusive. EV alone or combined with RA proved efficient in APL but no studies were conducted in the non-APL AML subtypes.

Herein, we investigated the potential efficacy of RA, EV, and ActD single agents or respective combinations *in vitro* and *in vivo*. Our main aim was to assess whether the sequential or simultaneous administration of RA followed by EV and/or ActD enhances cell death *in vitro* and confers a better therapeutic advantage *in vivo*.

CHAPTER IV

MATERIALS AND METHODS

A. Cell lines

Four cell lines were used for the *in vitro* studies: OCI-AML2 (Wild Type (WT) *NPM1*), OCI-AML3 (harboring the *NPM1* mutation), MOLM-13 (harboring the *FLT3* mutation), and THP-1 (lacking *p53*) (Table 5). OCI-AML2 and OCI-AML3 cells were grown in minimum essential medium α (MEM α) (Gibco) supplemented with 20% fetal bovine serum (FBS) (Sigma Aldrich) and 5% penicillin-streptomycin solution (Biowest), while MOLM-13 and THP-1 cells were grown in RPMI 1640 medium (Sigma Aldrich) supplemented with 10% FBS (Sigma Aldrich) and 5% penicillin-streptomycin solution (Biowest).

Table 5. Summary and characteristics of the used AML cell lines.

Cell line	Description	Origin	Morphology	P53	FLT3	NPM-1
OCI-AML2	Acute myeloid leukemia	Peripheral blood, Male, 65-year-old	Single, round to oval cells	Wild type	Wild type	Wild type
OCI-AML3	Acute myeloid leukemia	Peripheral blood, Male, 57-year-old	Single, round to oval cells	Wild type	Wild type	Mutant
MOLM-13	Acute monoblastic/ monocytic leukemia	Peripheral blood, Male, 20-year-old	Large, round, colony-forming morphology	Wild type	Mutant	Wild type
THP-1	Acute monocytic leukemia	Peripheral blood, Male, 1 year-old	Large, round, colony forming morphology	Null	Wild type	Wild Type

B. Drugs preparation

ActD stock was prepared by dissolving powder solution of ActD (Cosmegen® Lyovac) in sterile double distilled water while the light inside the biosafety cabinet level 2 (BCL2) was turned off, to obtain a stock concentration of 0.5 mg/ml. ActD stock was aliquoted and stored at -20ºC. For *in vitro* experiments, ActD was diluted in RPMI 1640 media to obtain a final concentration of 5 nM. For *in vivo* experiments, ActD was freshly diluted in 1X PBS to obtain a concentration of 0.06 mg/kg, and before the administration to mice.

EV stock was prepared by dissolving powder solution of EV (Sigma Aldrich) in DMSO under light sensitive conditions to obtain a stock concentration of 5 mM, aliquoted and then stored at -20ºC. For *in vitro* experiments, EV was diluted in RPMI 1640 media to obtain a final concentration of 1 µM. For *in vivo* experiments, EV was diluted in Lipofundin to obtain a final concentration of 2.5 mg/kg.

Retinoic Acid (RA) stock (6.6×10^{-2} M) (Sigma Aldrich) was stored in amber tubes at -80ºC. For *in vitro* experiments, RA was diluted in RPMI 1640 media to obtain a final concentration of 1 µM. For *in vivo* experiments, RA was diluted in 1X PBS with 5% ethanol (95%) and 5% Cremophor to obtain a concentration of 2.5 mg/kg.

All *in vitro* experiments were performed under yellow lights or while the visible light inside the BCL2 was turned off.

C. Cell viability assay

AML cells were seeded into 6-well plates at a concentration of 5×10^5 cells/1.5ml. Cells were treated with final concentrations of 1 µM of EV (Sigma Aldrich), 1 µM of RA, (Sigma Aldrich) and 5 nM of ActD (Cosmegen® Lyovac). To

examine the effect of RA before administration of EV and or ActD, cells were treated with RA for 24 hours before adding EV and Act as single agents or as a combination. Cell proliferation was evaluated at three different timepoints (24 h, 48 h and 72 h) using the trypan blue exclusion dye assay, according to the formula cells/ml = average number of cell counts x dilution factor x 10^4. Experiments for all conditions were conducted for at least three independent times, while for the triple combination EV/RA/ActD, only two independent experiments were performed.

D. Western blot analysis

Apoptosis pathways were investigated using Western blot analysis. Cells were washed in 1X PBS and solubilized at 4ºC using 2x Laemmli lysis buffer and stored at -20ºC. To determine the apoptotic proteins activated post-treatment, the primary antibodies used were anti-p53 (mouse monoclonal, Cat# 2524, Cell Signaling), anti-Pp53 (rabbit monoclonal, Cat# 9284, Cell Signaling), anti-p21 (rabbit monoclonal, 12D1, Cat# 2947, Cell Signaling), anti-Caspase 3 (rabbit polyclonal, H-277 SC-7272, Santa Cruz), and anti-PARP (rabbit polyclonal, SC-7150, Santa Cruz). 100 μg of proteins were loaded into 10% SDS-polyacrylamide gels for p53, P-p53 and p21, 12% gels for Caspase and 8% gels for PARP, then transferred into nitrocellulose membranes. Membranes were incubated overnight or over the weekend with the primary antibodies then washed with 1X PBS. Blots were then incubated with secondary antibodies mouse anti-rabbit (SC-2357, Santa Cruz) and mIgG mouse (SC-516102, Santa Cruz). Protein bands were visualized using Chemidoc® MP Imaging System (Biorad). Densitometry analysis were performed using ImageJ® software.

E. Xenograft animal models and *in vivo* treatments

NOD/Shi-scid IL2rγ-/- (NSG) mice were obtained from Jackson Laboratories (United States). All animal experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of AUB. All animals were housed in pathogen-free facility, and autoclaved water and food were administered regularly. Two million OCI-AML3 or OCI-AML2 cells were injected into the tail vein of 6- to 8-week-old mice.

Treatment with RA (2.5 mg/kg) began 10 days post engraftment of cell lines and lasted for 5 days during the second week. Mice were treated with EV (2.5 mg/kg) or ActD (0.06 mg/kg) as single agents or combined for 5 days during the third week. To test the effect of simultaneous administration of RA, a group of mice was treated with RA from days 17 to 22, along with the combination of EV/ActD (Figure 9).

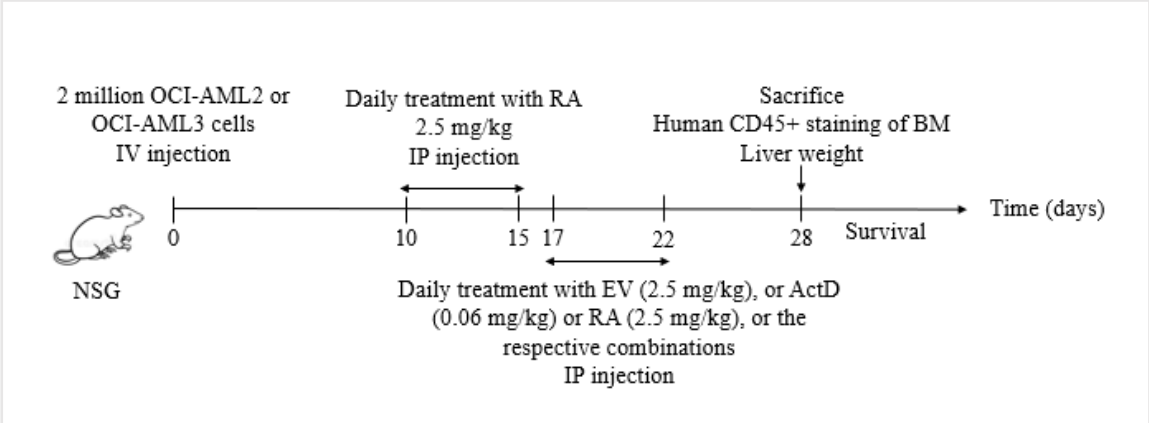


Figure 9. Timeline and treatment regimen *in vivo*.
Six to eight-week-old NSG mice were intravenously injected with either OCI-AML3 or OCI-AML2 cells. Mice were intraperitoneally treated with RA for 5 days, followed by EV, ActD as single agents or their combination. To compare the simultaneous administration of RA, EV and/or ActD with the sequential administration of RA first, followed by EV and/or ActD, a group of mice received RA/ActD/EV and was hence treated with RA during the second cycle of 5 days, along with ActD and EV. Mice were either monitored for survival or humanely sacrificed on day 28, liver weight was recorded, and BM samples were harvested from the tibia and femur of corresponding conditions and stained using human anti-CD45 antibody.

F. Survival assay, gross pathology, and organ harvesting

In each of the aforementioned treatment conditions (total n = 4-6 mice per treatment, number detailed in figure legends), some mice were monitored for overall survival and curves were plotted using GraphPad Prism 9. Some mice were humanely sacrificed on day 28 following deep anesthesia and subsequent cervical dislocation. Liver, spleen, kidney, and lungs were harvested and stored in formalin for future H&E staining and histopathology analysis. Liver weight was recorded and plotted.

G. Human CD45 staining

On day 28, BM cells were flushed from the femurs and tibias of euthanized animals. Cell surface staining was performed, in the dark, on 100 µl of the sample using 20 µl of PE mouse anti-human CD45 antibody (Cat# 555483 BD bioscience), for 45 minutes. Labeled samples were analyzed using a Guava flow cytometer.

H. Statistical analysis

All *in vitro* experiments were conducted for at least three independent experiments, except for the proliferation of the triple combination condition, which was performed twice for OCI-AML2 and OCI-AML3, while not performed for MOLM-13 and THP-1. Data was reported as mean ± standard. Significance levels (P-values of unpaired 2-tailed t-tests) were determined using Microsoft Excel 2012. Statistical significance was presented as follows: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. For *in vivo* experiments, Kaplan-Meier survival curves were plotted using GraphPad Prism 9.

CHAPTER V

RESULTS

A. The combinations of EV/ActD and EV/RA induce growth inhibition in AML cell lines

Initially, we investigated the effect of the simultaneous administration of EV, RA, and ActD as single agents or combined. Based on previous experiments (either published or conducted in our laboratory), EV (1 µM), RA (1 µM), and ActD (5 nM) were selected as the optimized working concentrations for all *in vitro* experiments.

In OCI-AML2 (wt-NPM1), we showed that RA alone moderately reduced OCI-AML2 viability at 72 h post-treatment ($P = 0.001$). This decrease reached around 40% when RA was combined with EV ($P < 0.001$). Moreover, ActD as single agent exhibited a prominent efficacy and decreased OCI-AML2 proliferation to around 20% at 72 h ($P < 0.001$), similar outcome was observed when RA was added to ActD showing that this is due to ActD alone (20% at 72 h $P < 0.001$). When ActD was combined with EV, the decrease was more prominent and reached 10% of viable cells only ($P < 0.001$). Addition of RA to the double combination of EV/ActD did not further reduce cell viability (Figure 10A). Our results demonstrate that OCI-AML2 cells are particularly sensitive to ActD, and the cell death phenotype is seemingly due to this drug. Adding EV to ActD slightly induces further cell death. However, adding RA to their combination or to ActD alone did not confer any additional benefit, as compared to ActD or ActD/EV (Figure 10A).

Similar results were obtained in OCI-AML3 having the *NPM1* mutation, where RA decreased cell growth to 50% 72 h post-treatment ($P < 0.001$) (Figure 10B), and

further reduction was observed when EV was added to RA to reach around 40% at 72 h ($P < 0.001$). Importantly, ActD as single agent potently inhibited cell proliferation, and near complete inhibition was observed at 72 h ($P < 0.001$). Nonetheless, the double combinations ActD/RA and EV/ActD or the triple combination EV/RA/ActD yielded the same results obtained with ActD single agent, demonstrating that the cell growth arrest is mostly the effect of ActD in the *NPM1* mutant AML cells (Figure 10B).

In MOLM-13 having *FLT3* mutation, at 72 h post-treatment, RA or EV single agents decreased cell viability to around 60% ($P < 0.01$ and $P < 0.05$, respectively) (Figure 10C). Strikingly, RA/EV combination resulted in growth suppression reaching around 20% of control values at 72 h ($P < 0.001$), and presumably indicating a synergistic effect. ActD alone or combined with EV resulted in a similar time-dependent decrease in viability with near complete abolishment at 72 h (Figure 10C), demonstrating that this cell death is mostly the effect of ActD. Our results demonstrate that in *FLT3* mutant AML cells, ActD is the most potent drug. Nonetheless, whether adding RA to ActD or to EV/ActD induces cell death at earlier time points remains to be investigated.

Lastly, in THP-1 cells which lack *p53*, EV and ActD as single agents moderately decreased growth at 72 h to around 70% ($P < 0.01$ and $P < 0.05$, respectively), while the EV/ActD combination significantly reduced viability to reach around 40% at 72 h ($P < 0.001$). RA as single agent, slightly decreased THP-1 viability, while combining RA with EV enhanced this reduction to reach around 40% at 72 h ($P < 0.001$) (Figure 10D). Our observations indicate that, in absence of p53, the combination of EV to either RA or ActD may lead to same beneficial effects. Whether RA/ActD or

the triple combination EV/RA/ActD may confer additional advantages remains to be explored.

Altogether, our results confirm a difference in therapeutic response according to mutations and reflect the therapeutic potency of ActD alone in cells with intact *p53*, or its combination with EV in OCI-AML2 and THP-1 *in vitro*. Moreover, adding RA to EV demonstrated some beneficial effect, but that effect was not as prominent as ActD alone or ActD combined with EV.

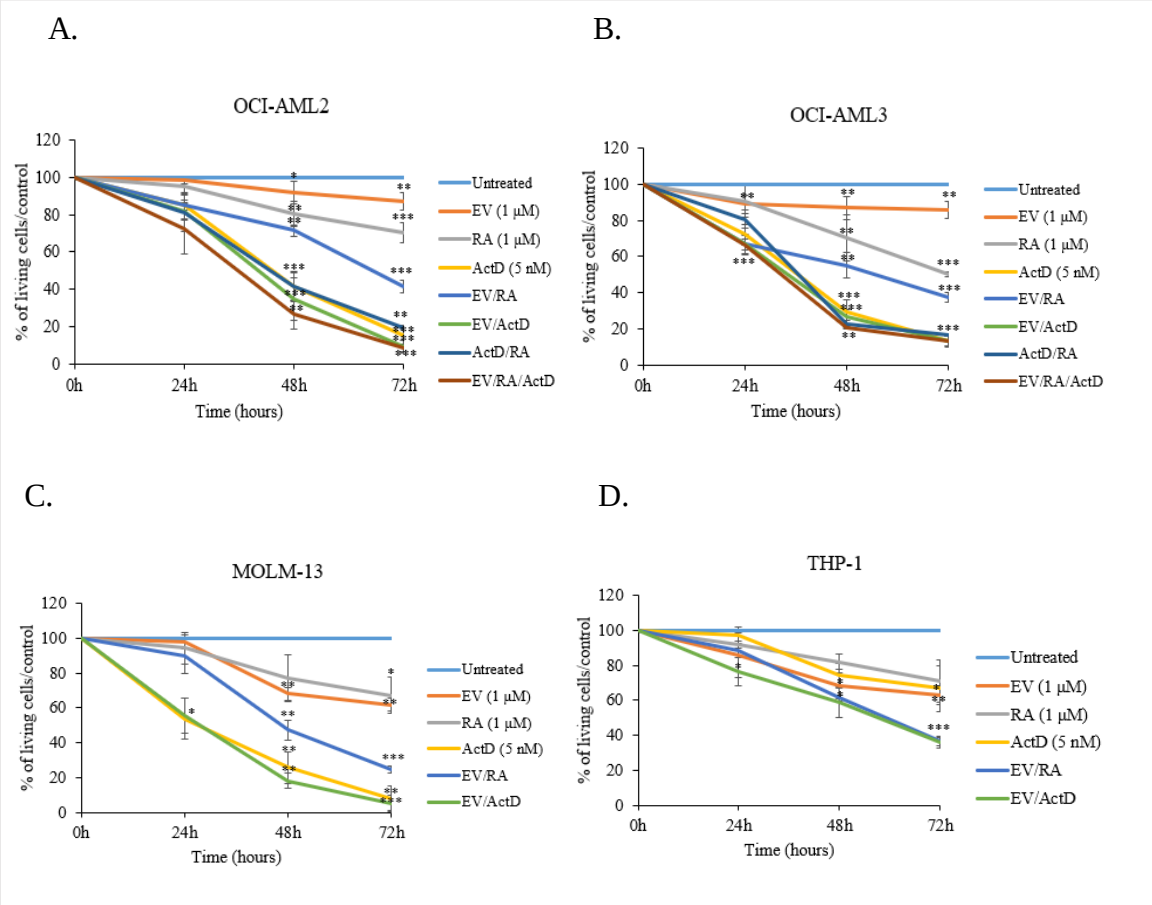


Figure 10. Combination treatments of EV/RA and EV/ActD induce growth inhibition in AML cell lines.
Trypan blue assay of A) OCI-AML2, B) OCI-AML3, C) MOLM-13 and D) THP-1 treated with EV (1 µM), RA (1 µM) and ActD (5nM) as single agents or combined. Four independent experiments were performed for OCI-AML2 and OCI-AML3 (except EV/RA/ActD and ActD/RA, n=2), and three for MOLM-13 and THP-1, and results

represent the average and are expressed as percentage of living cells/control (± SD) (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$).

B. Initial treatment with RA for 24 h followed by EV and/or ActD yielded the same efficacy than ActD/EV in inducing cell death *in vitro*.

We then explored whether an initial treatment with RA for 24 h, followed by EV and/or ActD yields a more pronounced cell death phenotype *in vitro*.

Our results indicate that a 48 h treatment with EV, following RA treatment, leads to around 50% cell death in OCI-AML2 ($P < 0.01$) (Figure 11A). This result was better than the co-administration of RA/EV that led to a moderate effect on cell death (around 20%, 48 h post-treatment) (Figure 10A). Strikingly, 48 h treatment with ActD, following RA treatment, led to a pronounced cell death (around 70%, $P < 0.001$) (Figure 11A). Yet, the effect of the single treatment with ActD was conferred and was more prominent at the same time point (Figure 10A). An original RA treatment for 24 h, followed by ActD and EV led to a decrease of 80% in cell viability at 72 h ($P < 0.01$) (Figure 11A). However, compared to our previous simultaneous treatment, this effect was less prominent than that obtained with EV/ActD or ActD alone (Figure 10A).

In OCI-AML3, initial administration of RA, followed by ActD, EV single agents or their combination led all to a pronounced and significant decrease in cell viability. RA/ActD, RA/EV or RA/ActD/EV reduced cell viability to around 20-30% at 72 h ($P < 0.001$, $P < 0.001$ and $P < 0.01$, respectively) (Figure 11B). Same percentage of viable cells was recorded previously after treatment with ActD alone or the triple combination EV/RA/ActD (Figure 10B), indicating that ActD alone can be as promising as the simultaneous or sequential RA, and/or EV/ActD treatment in the *NPM1-c* AML subtype. In other words, our results reflect the lack of any significant

difference between the two RA treatment regimens *in vitro*, yet proving once again and in line with prior research the efficacy of ActD in the *NPM1* mutant AML cells [84].

In MOLM-13 cells, an initial treatment with RA, followed by ActD alone led to decrease of MOLM-13 viability to reach a near complete abolishment at 72 h ($P < 0.001$) (Figure 11C). Similar data were obtained when treatment with ActD/EV, but not with EV alone, was performed ($P < 0.001$) (Figure 11C), reflecting that the observed effect is mainly due to ActD. Similar results for both conditions were previously seen following simultaneous administration of RA and EV or ActD (Figure 10C). These results confirm the lack of any advantage of treatment with RA, whether sequentially or simultaneously with ActD in AML cells with *FLT-3* mutation, and proving the efficacy of ActD alone in this subtype of AML.

Lastly, in the p53 null THP-1 cells, an initial treatment with RA followed by either EV or ActD did not yield to any significant or pronounced cell death (Figure 11D). RA treatment followed by ActD/EV led to a significant decrease of around 60% in cell viability ($P < 0.001$) (Figure 11D). However, same results were obtained when THP-1 cells were treated with EV/ActD without prior treatment with RA (Figure 10D). These results demonstrate that the use of RA as prior treatment does not result in additional inhibition in THP-1 proliferation. This outcome was somehow expected in these p53 null cells, given that the mechanism of action of both RA and ActD is mediated through p53 [73, 103].

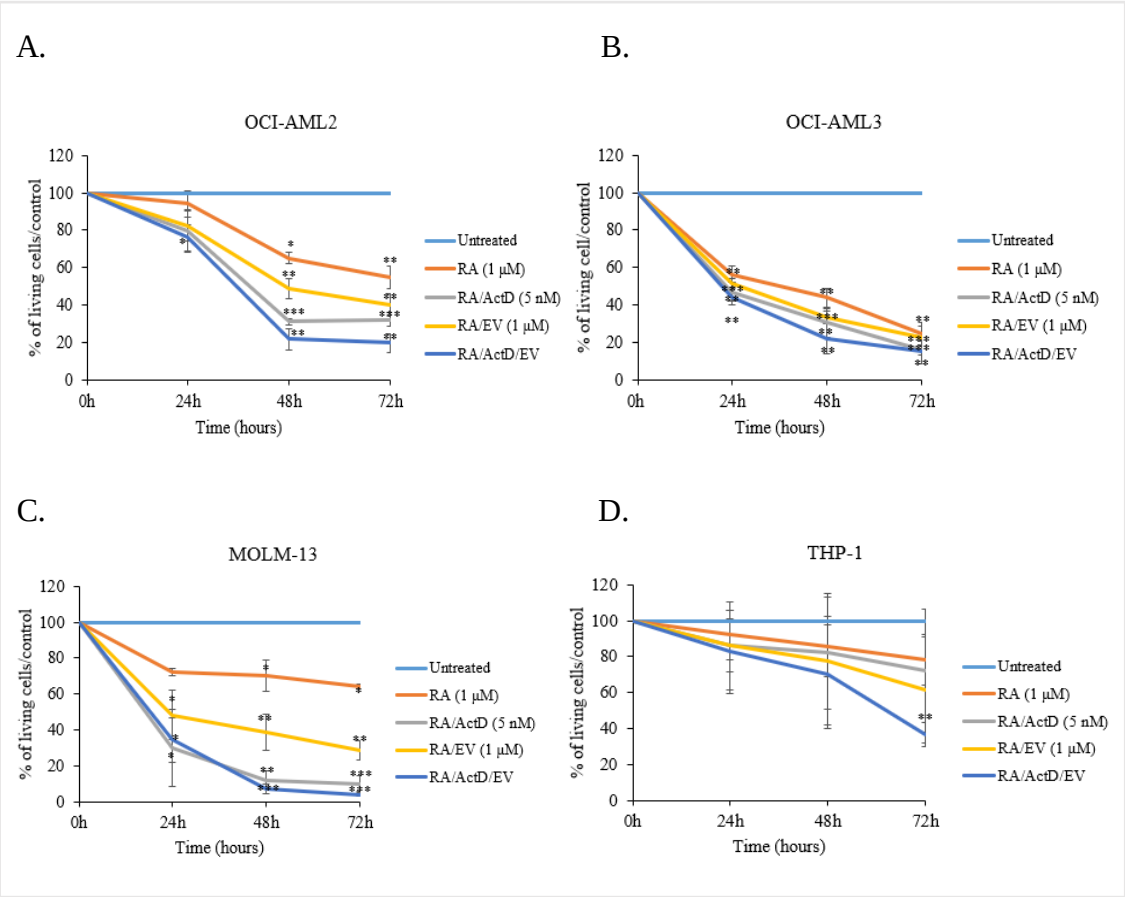


Figure 11. No advantage in reducing AML cellular growth is conferred after EV and ActD treatments following initial treatment with RA.
Trypan blue assay of A) OCI-AML2, B) OCI-AML3, C) MOLM-13 and D) THP-1 initially treated with RA (1 µM) for 24h, then with ActD (5 nM), EV (1 µM) or their respective combination. Three independent experiments were performed for OCI-AML2 and OCI-AML3 and two experiments for MOLM-13 and THP-1. Results represent the average and are expressed as percentage of living cells/control (± SD) (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$).

Collectively, our results presumably demonstrate in most tested AML cells, that RA treatment, whether administered before EV/ActD or simultaneously with this combination does not confer significant additional advantage, as compared to EV/ActD. Hence, in the following sections, we characterized the molecular mechanisms of cell death *in vitro*, using only the two cell lines, OCI-AML2 and OCI-AML3, 48 h post-treatment with ActD, EV, or their combination.

C. The combination of EV/ActD activates the p53 signaling pathway in OCI-AML2 and OCI-AML3 cells.

During apoptosis, p53 is upregulated and phosphorylated into its active form phospho-p53 (P-p53) [75] and activates its downstream effector p21 [104]. To investigate whether EV/ActD induced cell death *via* activation of the p53 apoptotic pathway, OCI-AML3 or OCI-AML2 cells were treated with EV (1 µM), ActD (5 nM) or EV/ActD for up to 48 h.

The activation of the p53 pathway was obtained in OCI-AML2 as reflected by the upregulation of the P-p53 levels with ActD, but not EV alone, and to a higher extent the combination ActD/EV (Figure 12). This is consistent with our previous observation, whereby we showed that OCI-AML2 growth is reduced to 20% at 72 h following ActD alone, and this reduction reaches a further 10% when EV is added to ActD (Figure 10A).

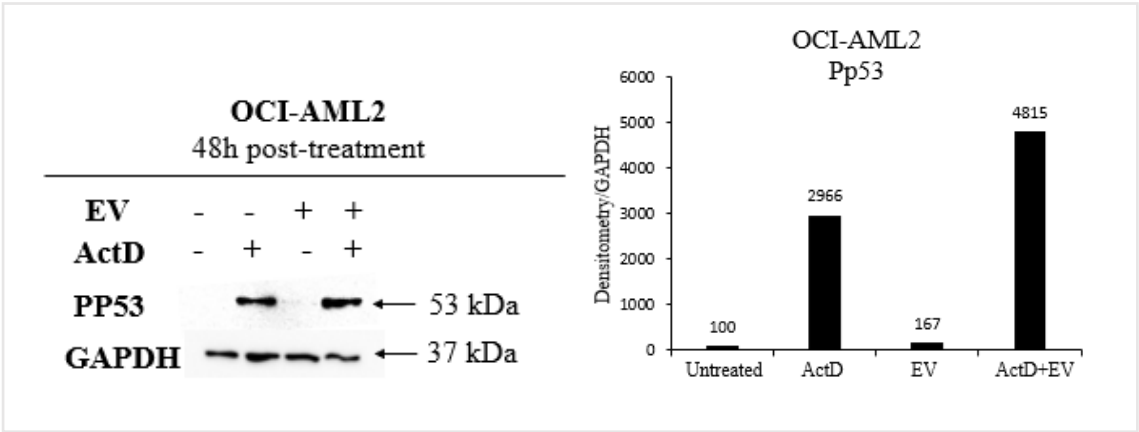


Figure 12. EV/ActD combination treatment activates p53 in OCI-AML2 cells. Western blot analysis of Pp53 in OCI-AML2 48 h post treatment. Cells were seeded at a concentration of $2x10^5$ cells/ml and treated with EV (1 µM) and/or ActD (5 nM). Cells were then collected at 48 hours post-treatment and protein lysates were immunoblotted against Pp53 antibody. Equal protein loading was ensured by re-probing the blots using GAPDH antibody. Black histograms represent the densitometry analysis of the ratio Pp53/GAPDH. Results are from one experiment.

In OCI-AML3 cells, ActD but not EV, induced the expression of p53 and p21 proteins as single agents. Interestingly, both p53 and p21 were markedly upregulated when ActD was combined with EV (Figure 13), reflecting a potential synergy of these two agents on the p53 pathway activation in OCI-AML3 cells. However, unlike our growth inhibition experiments, where we showed that ActD, whether alone or combined with EV, reduces OCI-AML3 viability by similar amounts at 48 h (Figure 10B), the effect of the combination on the activation of the p53 pathway seemed to be more prominent.

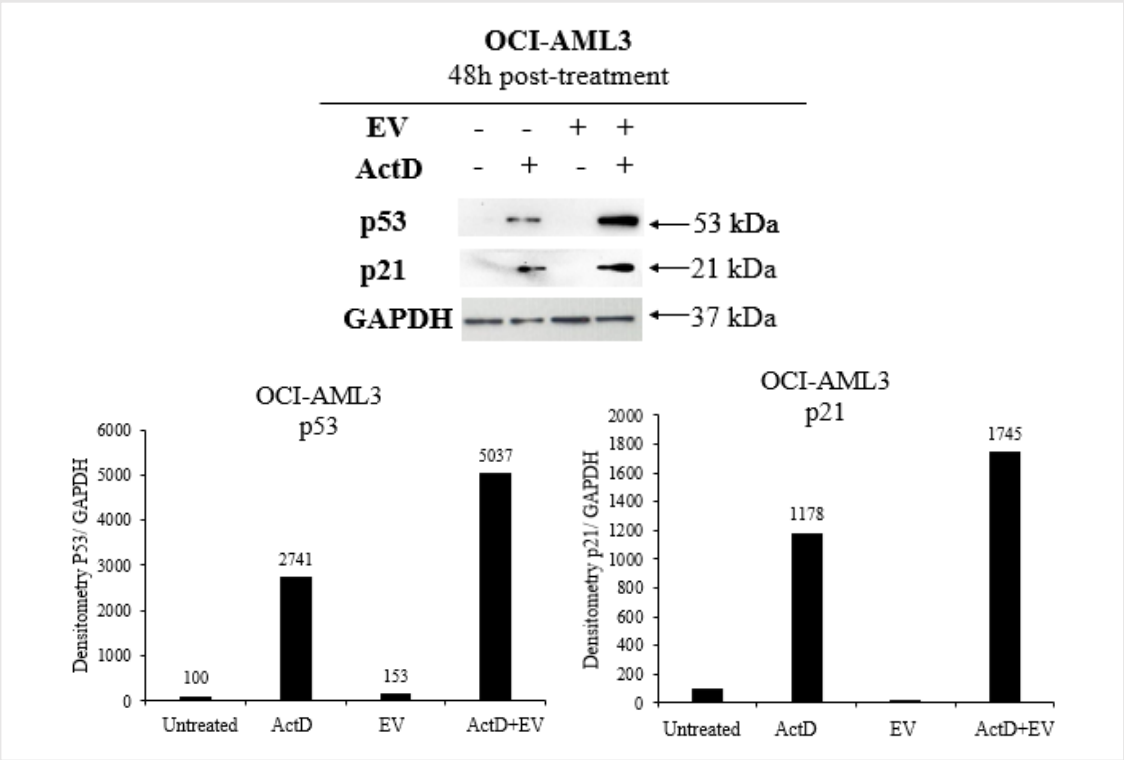


Figure 13. EV/ActD activate p53 signaling pathway in OCI-AML3 cells.
Western blot analysis of p53 and p21 in OCI-AML3 48 h post treatment. Cells were seeded at a concentration of $2x10^5$ cells/ml and treated with EV (1 µM) and/or ActD (5 nM). Cells were then collected at 48 h post-treatment and protein lysates were immunoblotted against p53 or p21 antibodies. Equal protein loading was ensured by re-probing the blots using GAPDH antibody. Black histograms represent the densitometry analysis of p53 and p21 as protein level/GAPDH. Results are from one experiment.

D. The combination of EV/ActD induces a caspase-dependent mechanism of apoptosis in OCI-AML2 cells.

A hallmark of p53-induced apoptosis is the cleavage of caspases [105] and PARP [106]. Indeed, activated caspases 3 and 7 lead to a subsequent cleavage of PARP-1 (116 kDa), in fragments of 89 and 24 kDa, during apoptosis which has become a useful hallmark of apoptosis [107, 108]. To investigate whether EV and/or ActD induce cleavage of both caspase 3 and PARP, OCI-AML2 cells were treated with EV, ActD or EV/ActD for up to 48 h. Our results show a caspase-dependent mechanism of apoptosis as demonstrated by cleavage of procaspase 3 (~35 kDa) into its active form (~17 kDa) following treatment with ActD or the combination EV/ActD (Figure 14). PARP was also cleaved in OCI-AML2 at 48 h following treatment with ActD single agent, and to a higher extent when EV was added to ActD. These results confirm that EV/ActD exhibit a potential synergy between EV and ActD in inducing caspase-dependent mechanism of apoptosis (Figure 15).

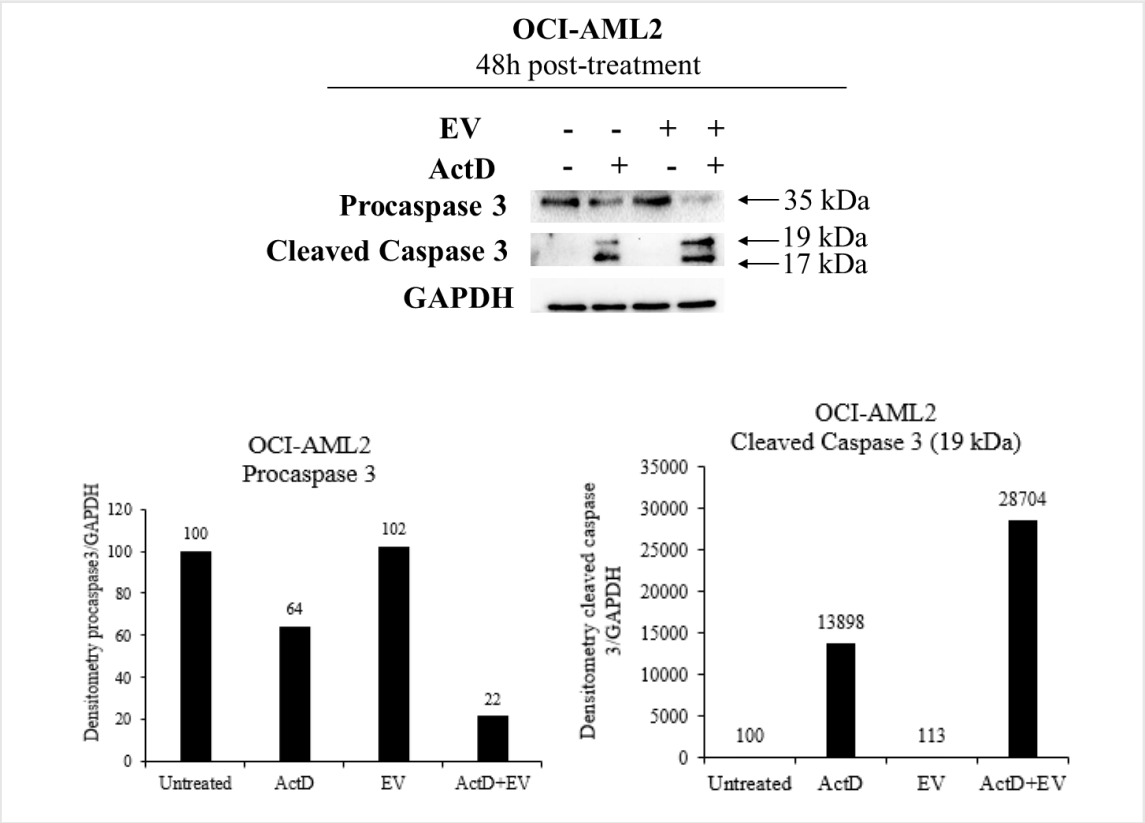


Figure 14. EV/ActD induce a caspase-dependent mechanism of apoptosis in OCI-AML2 cells.
Western blot analysis of procaspase 3 and cleaved caspase 3 in OCI-AML2 48h post treatment. Cells were seeded at a concentration of $2x10^5$ cells/ml and treated with EV (1 µM) and/or ActD (5 nM). Cells were then collected at 48 h post-treatment and protein lysates were immunoblotted against caspase 3 antibody. Equal protein loading was ensured by re-probing the blots using GAPDH antibody. Black histograms represent the densitometry analysis of procaspase 3 and cleaved caspase 3 as the ratio protein level/GAPDH. Results are from one experiment.

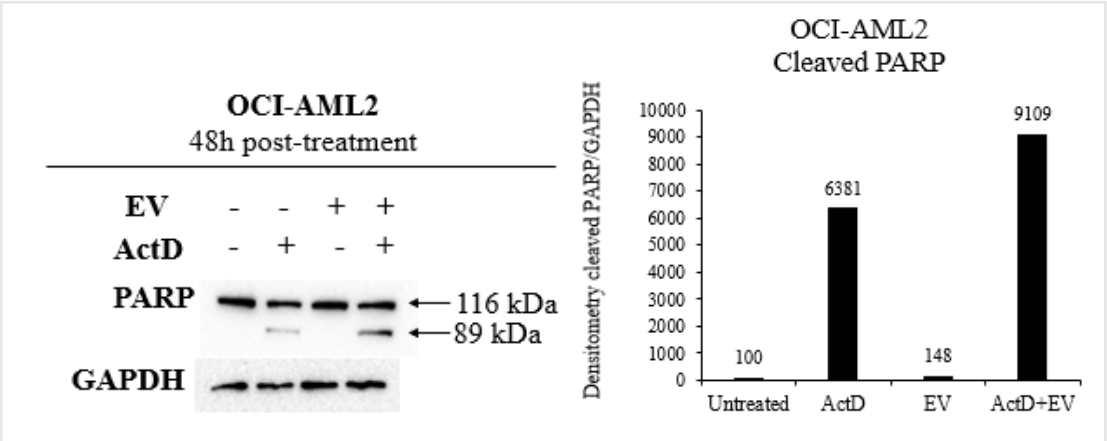


Figure 15. Induction of PARP cleavage in OCI-AML2 after EV/ActD treatment.

Western blot analysis of PARP and cleaved PARP in OCI-AML2 48h post treatment. Cells were seeded at a concentration of $2x10^5$ cells/ml and treated with EV (1 µM) and/or ActD (5 nM). Cells were then collected at 48 h post-treatment and protein lysates were immunoblotted against PARP antibody. Equal protein loading was ensured by re-probing the blots using GAPDH antibody. Black histograms represent the densitometry analysis of the ratio cleaved PARP/GAPDH. Results are from one experiment.

E. Initial treatment with RA followed by EV/ActD confers a slight beneficial survival advantage in OCI-AML2 xenograft mouse models.

In vitro screening for antitumor activity using AML cell lines has several limitations including the absence of the appropriate cancer microenvironment and failure to predict the effect of treatment *in vivo*. Thus, we used two AML xenograft mouse models injected with OCI-AML2 and OCI-AML3 to investigate the effect of sequential or simultaneous treatment of RA with EV and/or ActD. We followed the *in vivo* timeline described in Figure 9. Untreated animals were euthanized following tremendous deterioration in their health status, and sacrificed on day 28 along with a few mice from other conditions to harvest their organs and perform BM flushing from tibias and femurs.

Our preliminary results on the limited number of tested mice, demonstrate that in OCI-AML2 xenograft mice, EV or ActD as single agents prolonged survival beyond 55 days, while mice treated with this combination succumbed at day 45. This is contradictory to *our vitro* results where we demonstrated that the combination of EV/ActD inhibits OCI-AML2 more potently than single agents (Figure 10A). Additionally, median survival of mice treated with RA prior to EV or ActD alone was increased from 40 days and 33 days respectively to 55 days following EV/ActD, presumably indicating that RA prior to EV/ActD might be of benefit in this subtype of

AML. Yet these preliminary results require further investigation on a higher number of mice (Figure 16).

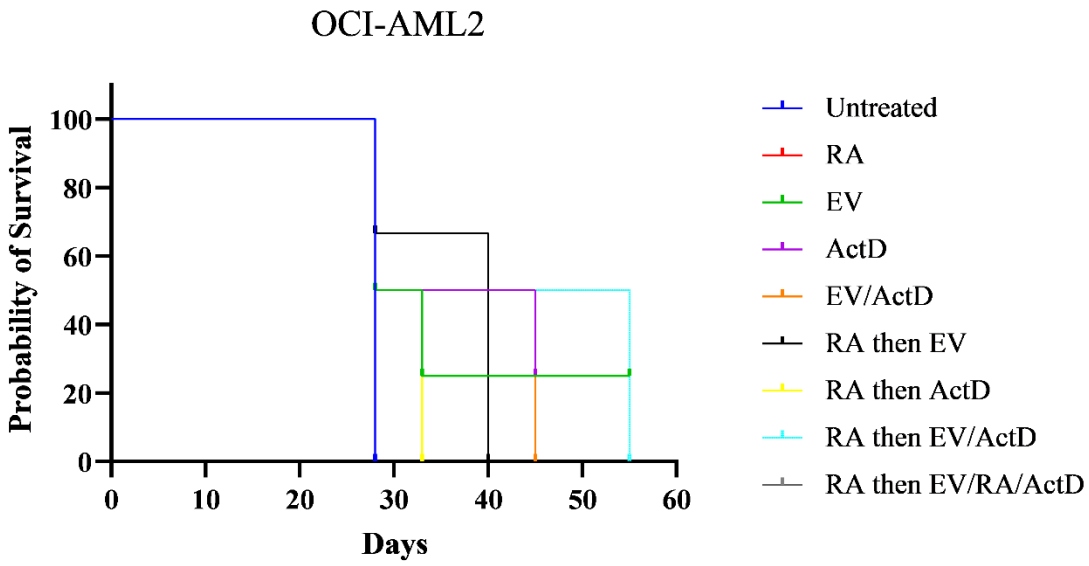


Figure 16. Kaplan-Meier overall survival plot of OCI-AML2 xenograft mouse models. Survival curve was plotted using GraphPad Prism 9 software. On day 28 some groups were euthanized following a major deterioration in their health status and sacrificed to harvest organs and perform BM flushing: untreated (n=6), RA (n=4), EV (n=2), ActD (n=2), EV/ActD (n=2), RA first then EV (n=1), RA first then ActD (n=2), RA first then EV/ActD (n=2), RA first then EV/RA/ActD (n=4). Mice monitored for survival: EV (n=2), ActD (n=2), EV/ActD (n=2), as well as mice initially treated with RA then treated with the following treatments: ActD (n=2), EV (n=2), EV/ActD (n=2).

F. EV single agent or RA prior to EV/RA/ActD increases survival of OCI-AML3 xenograft mouse models.

In OCI-AML3 xenograft mice, without prior administration of RA, our preliminary results indicate that the median survival of mice treated with EV or ActD alone was 49 and 35 days, respectively, whilst animals that received RA first then EV or ActD survived for 34 days and 31 days, respectively. This is in line with or previous results where we demonstrated the lack of advantage of prior RA treatment in OCI-AML3 (Figure 11B). Moreover, the median survival of mice treated with EV/ActD

combination was 34 days, similar to ActD alone, suggesting once again that anti-leukemic effect in *NPM1*-mutated AML is mainly due to ActD alone (Figure 10B). Original treatment for 5 days with RA followed by EV/RA/ActD for an additional 5 days, prolonged survival by 4 days only (median survival 38 days). These preliminary observations indicate that EV/ActD, as well as the initial treatment with RA before administration of the indicated drugs confer no survival advantage in OCI-AML3 mice. Yet, EV as single agent might be beneficial (Figure 17).

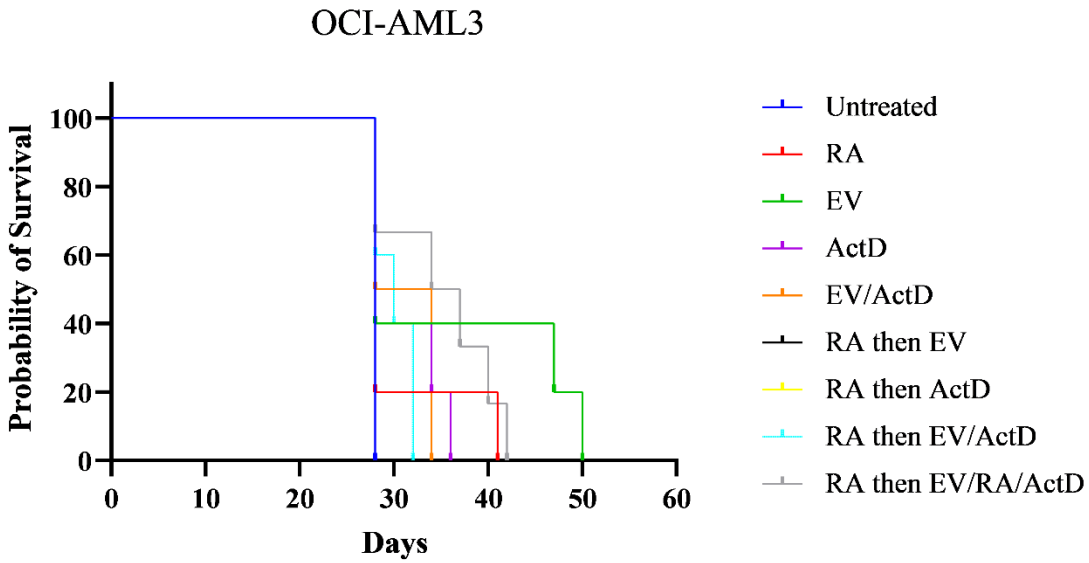


Figure 17. Kaplan-Meier overall survival plot of OCI-AML3 xenograft mouse models. Survival curve was plotted using GraphPad Prism 9 software. On day 28 some groups were euthanized following a major deterioration in their health status and sacrificed to harvest organs and perform BM flushing: untreated (n=5), RA (n=1), EV (n=3), ActD (n=3), EV/ActD (n=3), EV/RA/ActD (n=2), RA first then EV (n=3), RA first then ActD (n=4), RA first then EV/ActD (n=2). Mice monitored for survival: RA (n=1), EV (n=2), ActD (n=2), EV/ActD (n=3), as well as mice initially treated with RA then treated with the following treatments: ActD (n=1), EV (n=2), EV/ActD (n=3) and EV/RA/ActD (n=4).

G. Initial treatment with RA followed by EV and/or ActD does not exhibit any beneficial effect on liver weight in AML xenograft mouse models.

Blast infiltration is reported in AML patients, resulting in hepatomegaly and liver failure [109]. Therefore, to better understand the effect of our treatments on AML xenograft mice, a group of xenograft mice were sacrificed on day 28. Liver was harvested and weighed, and no reduction in mass was observed in both OCI-AML2 mice (Figure 18) and OCI-AML3 mice (Figure 19), suggesting that original treatment with RA followed by EV and/or ActD does not reduce AML induced hepatomegaly.

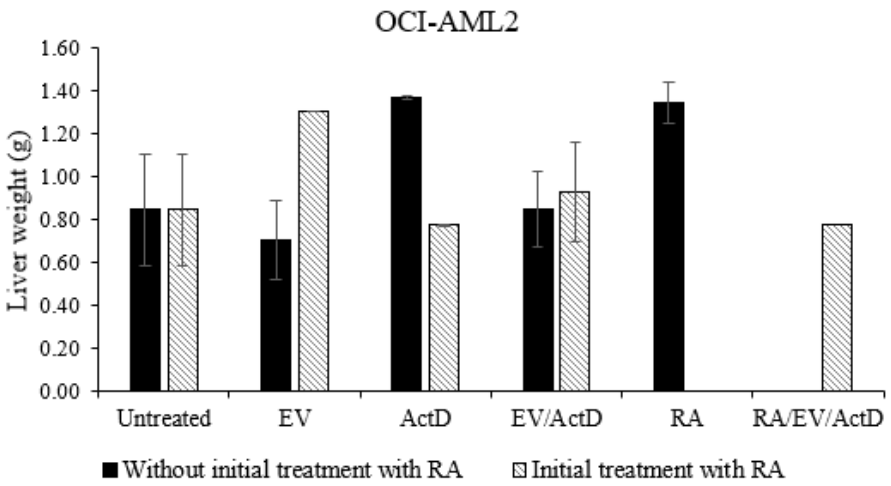


Figure 18. Liver weight of OCI-AML2 xenograft mice following RA, or EV and/or ActD, or subsequent to initial treatment with RA.
Black histograms show liver weight harvested from OCI-AML2 mice treated with the indicated drugs without initial RA treatment (Untreated (n=6), RA (n=4), EV (n=2), ActD (n=2), EV/ActD (n=2)), and white histograms show liver weight of animals treated with RA for one week followed by the indicated treatments (EV (n=1), ActD (n=2), EV/ActD (n=2), RA/EV/ActD (n=4)). Results are from one experiment.

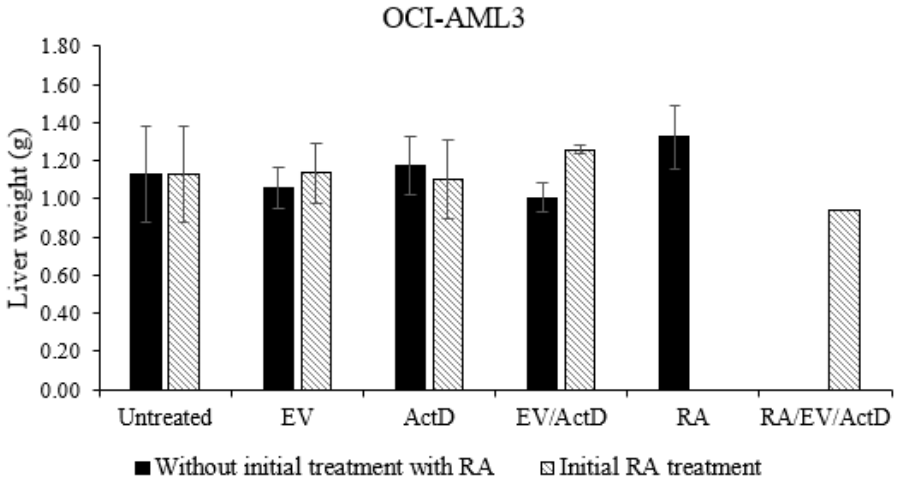


Figure 19. Liver weight of OCI-AML3 xenograft mice following RA, or EV and/or ActD, or subsequent to initial treatment with RA.
Black histograms show liver weight harvested from OCI-AML3 mice treated with the indicated drugs without RA pretreatment (untreated (n=5), EV (n=2), ActD (n=2), EV/ActD (n=3)), and white histograms show liver weight of animals treated with RA for 5 days followed by the indicated treatments (ActD (n=4), EV (n=3), EV/ActD (n=2), RA/EV/ActD (n=2)). Results are from one experiment.

H. Initial treatment with RA followed by EV seems to eradicate tumor burden in OCI-AML2 xenograft mouse models.

OCI-AML2 xenograft mice were sacrificed on day 28 and BM cells were flushed and stained for human CD45. Flow cytometry analysis revealed a very low engraftment in untreated controls as reflected by a low CD45 positivity (10%). Unfortunately, this leukemic burden markedly worsened following treatment with EV and/or ActD. Indeed, human DC45 positivity increased to reach around 30%, in the three conditions of treatment, reflecting a higher tumor burden. Strikingly, mice that received an initial treatment with RA, and were subsequently treated with EV witnessed a complete eradication of CD45+ leukemic cells. Animals treated with EV/ActD following original treatment with RA showed slight improvement, whereby OCI-AML2 burden was reduced to 6%. However, this reduction is very similar to the low engraftment of the untreated control. Furthermore, RA treatment for 5 days followed by

EV/ActD/RA for additional 5 days worsened leukemic burden tremendously, whereby CD45+ leukemic cells markedly increased to reach 60% (Figure 20). It is important to note that while clear reduction in OCI-AML2 burden is observed upon EV treatment subsequent to RA administration, BM flushed from only one mouse was stained for CD45+ and these preliminary results remain to be confirmed using a significant number of mice. Hence, our results validate that the addition of RA as initial treatment has no efficacy in treatment of AML, and cement our previous *in vitro* observations. A single exception was observed which is RA followed by EV but which require confirmation to draw a clear conclusion.

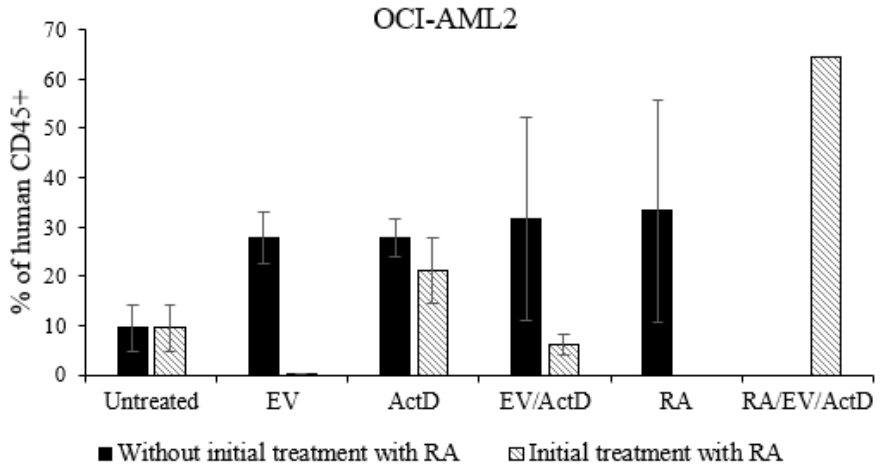


Figure 20. Percentage of human CD45 positivity in OCI-AML2 xenograft mice RA, or EV and/or ActD, or subsequent to initial treatment with RA.
Black histograms represent the percentage of human CD45 in BM samples retrieved from OCI-AML2 mice treated with the indicated drugs without initial RA treatment (Untreated (n=6), RA (n=4), EV (n=2), ActD (n=2), EV/ActD (n=2)), and white histograms represent the percentage of human CD45 in BM samples harvested from OCI-AML2 mice treated with RA followed by the indicated treatments (EV (n=1), ActD (n=2), EV/ActD (n=2), RA/EV/ActD (n=4)). Results are from one experiment.

CHAPTER VI

DISCUSSION AND FUTURE PERSPECTIVE

Before discussing our preliminary results, it is worth noting that the unprecedented COVID19 pandemic and the consecutive lock-downs tremendously affected our work. We are fully aware that our results are very preliminary and necessitate, for most experiments, confirmation to reach n=3 *in vitro* and at least 7 to 10 mice per condition in *vivo*. However, in order to fulfill the MS thesis requirements, we will still discuss these preliminary results in light with the published literature.

AML is one of the most frequent and complex leukemias in adults. To date, and despite the advances of DNA sequencing techniques and the revolutionized personalized medicine, a variety of AML subtypes still represent an unmet medical condition, with either poor prognosis and high rates of relapse and resistance to standard chemotherapy, hence requiring more appropriate therapeutic regimens.

RA therapy inducing myeloid differentiation in APL, a subtype of AML, has been very successful and provides major benefits in eradicating the disease when combined with ATO [62]. Nevertheless, its efficacy in other subtypes of AML has been less studied [110]. Here, we investigated the impact of incorporating RA initially to induce differentiation, followed by treatment with EV, ActD, or their combinations, on non-APL AMLs.

Consistent with previous studies [73, 111], our initial results reveal a cell growth inhibition by RA single agent in *NPM1* mutant OCI-AML3 (Figure 10B). We then showed that the addition of EV to RA is more efficient than EV or RA single agents and

significantly reduces the viability of all tested cell lines, regardless of their karyotype (Figure 10). These observations are in line with a previous investigation which demonstrated the synergy between RA and EV in inducing growth arrest in APL cells [102] and demonstrate an efficacy of this combination in non-APL AML.

In line with prior research [84], our results indicate that ActD potently reduced AML cell proliferation (Figure 10A, 10B, 10C). This growth inhibition is less prominent in THP-1 cells lacking p53 (Figure 10D), highlighting a potential possible p53-independent mechanism of action of this drug [83, 84]. We showed a pronounced cell death in OCI-AML2, MOLM-13, and THP-1, when EV was added to ActD, while this combination yielded the same effect of ActD single agent in OCI-AML3 with *NPM1c* mutation (Figure 10B). This is consistent with the previously documented role of ActD alone in inhibiting *NPM1*-mutated AML [86]. Importantly, we documented an efficacy of this drug in MOLM-13 cells (Figure 10C), highlighting a potential benefit of ActD in the *FLT3*-mutants AML subtypes.

Initial treatment with RA *in vitro,* followed by administration of EV and/or ActD did not confer an additional advantage in reducing AML cell viability (Figure 11) as compared to the simultaneous treatments with these drugs (Figure 10). This unresponsiveness of AML cell lines to original treatment with RA could be attributed to aberrant epigenetic gene regulation and transcription in non-APL AML cells [112]. In that sense, RAR target genes may not be properly activated due to epigenetic promoter silencing of target genes or modification of transcription factors, therefore affecting their susceptibility to RA induced differentiation [57].

EV/ActD combination decreased cell viability to near complete inhibition in all tested cell lines (Figure 10). We demonstrated that ActD alone, or in combination with

EV, resulted in the activation of the p53 pathway (through either the upregulation of its downstream p21 player or through the upregulation of P-p53). Cleavage of caspase3 and PARP, indicative of apoptosis, were also noted, in OCI-AML2 and in OCI-AML3 cells (Figure 12,Figure 13, Figure 14, and Figure 15). Our observations are consistent with prior research which reported the role of low levels of ActD in inducing p53-dependent apoptosis in AML cell lines [82]. However, unlike our growth supression data in OCI-AML3, where the synergy could be barely noted with ActD/EV combination and where most of the inhibitory effect was due to ActD, our protein expression analysis revealed a synergistic effect of ActD/EV on p53 activation and apoptosis in this cell line (Figure 10B). Yet the results obtained in OCI-AML2 were coherent in both our proliferation analysis (Figure 10A), and our protein expression evaluation (Figures 12, 14, 15). In the analysis of the p53 activation pathway, the use of p53 and p21 antibodies in one cell line, and P-p53 in the other cell line were purely due to technical issues and shortage of antibody. We also faced delays in receiving western blot reagents and antibodies due to the economic crisis as well as delays in receiving reagents due to the COVID pandemic.

In vivo, prolonged survival of OCI-AML2 xenograft animal models was conferred following treatment with EV or ActD alone and to a lesser extent following original treatment with RA prior to EV/ActD, which cements our previous *in vitro* proliferation assay results where we demonstrated that administration of RA subsequent to EV/ActD markedly reduced OCI-AML2 viability (Figure 11A), yet the effect was not as prominent as EV/ActD or ActD alone (Figure 10A). However, in contrast with the survival results, the indicated drugs, whether administered sequentially or simultaneously with RA, failed to alleviate leukemic burden and treated mice presented

with sustained hepatomegaly (Figure 18) and a tremendous increase in leukemic blasts in the BM (Figure 20). Whilst the original treatment with RA followed by EV was better than the co-administration of RA/EV *in vitro* (Figure 11A) and reduced leukemic burden in the BM of OCI-AML2 mice *in vivo* (Figure 20), this combination did not confer any survival advantage (Figure 16), nor did it reduce hepatomegaly in treated animals (Figure 18). The inconsistency between *in vitro* and *in vivo* results requires additional investigation.

While ActD yielded potent growth inhibition of OCI-AML3 *in vitro* (Figure 10B), we revealed that it did not reduce hepatomegaly (Figure 19) and did not enhance survival of OCI-AML3 xenografted mice (Figure 17). This result is surprisingly inconsistent with previous clinical trials which demonstrated the clear benefit of using ActD in treatment of individuals with relapsed/refractory *NPM1-c* AML [85, 86]. Our *in vivo* results need to be confirmed on a higher number of mice before getting a clear conclusion. However, we demonstrated that RA treatment for 5 days followed by EV/ActD/RA for additional 5 days, enhanced survival of OCI-AML3 mice, to a lesser extent than EV as single agent (Figure 17), which is in contrast with our *in vitro* proliferation results whereby we showed that triple combination without initial administration of RA yields higher OCI-AML3 growth inhibition than EV alone (Figure 10B). Although a survival advantage was obtained, hepatomegaly and tumor burden were both persistent after treatment (Figure 19). The lack of a therapeutic benefit of RA alone or combined with other drugs is in line with previously reported meta-analysis where RA added to chemotherapy did not reveal any benefit on the overall survival of patients with non-APL AML [113].

In conclusion, our study reveals that the beneficial effect of sequential or simultaneous treatment with RA, EV and/or ActD is dependent on the AML mutation. For instance, our *in vitro* results demonstrate a beneficial effect of EV/RA in inducing cell death of *FLT*3 and *p53*-mutated AML, yet further molecular investigation of the p53 and the mTOR pathway is needed, particularly that synergy between EV/RA was reported in APL cells via upregulation of mTORC1 negative regulators [102]. More importantly, we revealed the potent effect of EV/ActD mostly on cells with intact p53. The mTOR pathway remains to be elucidated in these cells, to explain whether the observed synergy could be attributed to ActD, especially that several previous studies discussed a tight link between ribosome biogenesis and mTORC1 signaling [114-116]. Finally, it is worth noting that we witnessed discrepancies between our *in vitro* and *in vivo* data. However, the number of tested mice, in all conditions, did not exceed 4 mice per group, entailing the need of increasing the number of mice to draw confirmed conclusions.

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