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Effect of Imiquimod and derivatives compounds EAPB0203 and EAPB0503 on Leukemia/lymphoma models

Ali Nabbouh

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THÈSE

Pour obtenir le grade de
Docteur

Délivré par l'**Université de Montpellier**

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Et de l'unité de recherche **Institut des Biomolécules Max
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Spécialité : **Biologie Santé**

Présentée par **Ali Nabbouth**

**Effet de l'Imiquimod et de composés
dérivés EAPB0203 et EAPB0503 sur des
modèles de leucémies et de lymphomes**

Soutenue le **16 Décembre 2016** devant le jury composé de

Mme **Jihane Basbous**, CR, CNRS Montpellier

Mr **Pierre Cuq**, Pr, Université Montpellier

Mme **Hiba El Hajj**, Pr assistant,

Université Américaine de Beyrouth

Mr **Issam Kassab**, Pr associé, Université libanaise

Mme **Carine Masquéfa**, Pr, Université Montpellier

Mme **Anne-Françoise Tilkin-Mariamé**, DR,

Université Paul Sabatier de Toulouse

Examineur

Examineur

Codirecteur de thèse

Rapporteur

Directeur de thèse

Rapporteur



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List of abbreviations

Allo-SCT	Allogeneic Stem Cell Transplant	HSCT	Hematopoietic stem cell transplant
AML	Acute Myeloid Leukemia	IAP	Inhibitor of apoptosis protein
APL	Acute promyelocytic leukemia	KSP	Kinesin spindle protein
Ara-C	Cytarabine	LEN	Lenalidomide
CALGB	Cancer and Leukemia Group B	WHO	World Health Organization
CBF	Core binding factor	MDS	Myelodysplastic syndrome
CR	Complete remission	MMP	Mitochondrial membrane potential
CI	Comorbidity Index	MRD	Minimal residual leukemia
CN	Cytogenetically normal	MPD	Myeloid proliferative disorder
CNS	Central nervous system	NCRC	National Cancer Research Council
DAPI	4',6-diamidino-2-phenylindol	NPM1	Nucleophosmin
DMSO	Dimethylsulfoxide	NGS	Next Generation Sequencing
DNR	Daunorubicin	NOS	Not otherwise specified
DNMT3A	DNA methyltransferase 3 alpha	OS	Overall survival
ECOG	Eastern Cooperative Oncology Group	PARP	Poly(ADP-ribose)polymerase
ELN	European LeukemiaNet	PCR	polymerase chain reaction
FDA	Food and Drug Administration	PBS	Phosphate Buffered Saline
FAB	French–American–British	PI	Propidium iodide
FLT-3	Fms-like tyrosine kinase 3	SAKK	Swiss Group for Clinical Cancer Research
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	STAT-5	Signal Transducer and Activator of Transcription-5
HCT	Hematopoietic Cell Transplantation	UK	United Kingdom
HOVON	Haemato Oncology Foundation for Adults in the Netherlands		

Résumé de la thèse en Français

La leucémie myéloïde aiguë (LMA) est une maladie clonale hétérogène caractérisée par une prolifération des cellules myéloïdes immatures et une défaillance de la moelle osseuse. Malgré les avancées rapides dans le domaine de la LMA, notamment concernant de nouvelles cibles thérapeutiques et une meilleure compréhension des mécanismes biologiques, le traitement clinique de la LMA reste inchangé et dépend du caryotype des patients. Lors des trois dernières décennies, la plupart des patients ont fini par récidiver et décéder de la maladie; ce qui fait qu'il n'y a encore aucun schéma thérapeutique standard qui améliore le pronostic et le traitement de la LMA.

Les imidazoquinoxalines sont des dérivés de l'imiquimod qui présentent un effet immunomodulateur indirect et une activité anti-tumorale directe contre le mélanome et le lymphome à cellules T, provoquant l'inhibition de la croissance cellulaire et l'induction de l'apoptose par la voie dépendante des caspases. Nous avons étudié les effets des dérivés de la série imidazoquinoxaline, EAPB0203 et EAPB0503, sur des lignées de cellules humaines LMA. Nous avons montré que EAPB0503 inhibe la croissance des lignées cellulaires LMA dotées de la mutation *NPM-1* de manière dose et temps dépendants. De plus, EAPB0503 présente une activité inhibitrice plus forte que celle de l'EAPB0203 sur les cellules OCI-AML3 et IMS-M2. Un effet similaire était obtenu sur les blastes provenant des patients LMA ayant une mutation *NPM-1*. L'introduction de la *NPM-1* mutée dans les cellules ayant une *NPM-1* sauvage, les rend sensible à l'effet de l'EAPB0503.

De plus, EAPB0503 induit une apoptose massive comme démontré par l'analyse du cycle cellulaire avec une accumulation de cellules traitées en phase preG0. L'apoptose a été confirmée par la réponse positive au test de l'annexine V, le clivage de PARP et la dissipation du potentiel membranaire mitochondrial dans les cellules OCI-AML3 traitées.

EAPB0503 a augmenté les niveaux d'expression et de phosphorylation de p53 et de la protéine p21 qui est en aval de p53.

Au niveau moléculaire, EAPB0503 induit la dégradation protéasomique de la protéine NPM-1 mutée, ainsi qu'un rétablissement de la localisation nucléolaire de la NPM-1 sauvage. En outre, EAPB0503 a montré une réduction sélective du nombre de blastes dans la moelle osseuse *in vivo*, dans des animaux immunodéprimés xéno greffés avec des cellules AML dotées de la mutation *NPM-1*.

Ces résultats sélectifs du composé EAPB0503 dans les cellules LMA présentant la mutation *NPM-1*, renforcent l'idée d'un ciblage de l'oncoprotéine NPM-1 mutée pour éliminer les cellules leucémiques et justifient une évaluation préclinique plus large puis une évaluation clinique de ce composé.

En conclusion, nos études mettent en évidence l'utilisation de l'EAPB0503 comme un candidat médicament prometteur qui présente une activité anti-tumorale encourageante et qui devrait faire l'objet d'études précliniques dans le cadre d'une thérapie ciblée contre la LMA.

Summary of the thesis in English

Acute myeloid leukemia (AML) is a heterogeneous clonal disorder characterized by an immature myeloid cell proliferation and bone marrow failure. Although remarkable improvements in the field were achieved in regards of characterizing new drug targets and better understanding of the biology of the disease, the clinical treatment of AML remains unchanged and largely depends on the karyotype of patients. In addition, the majority of AML patients relapse and die of the disease. Thus, until nowadays, there is no standard regimen that yet improves prognosis and treatment of AML.

Imidazoquinoxalines are imiquimod derivatives with indirect immunomodulatory effect and direct antitumor activity on melanoma and T-cell lymphoma, attributed to growth inhibition and induction of apoptosis through caspase-dependent pathway. We examined the effects of imidazoquinoxaline derivatives, EAPB0203 and EAPB0503, on human AML cells. We found that EAPB0503 inhibit cell growth of AML cell lines that harbors the *NPM-1* mutation in a time- and dose-dependent manner. Compared to the previously synthesized EAPB0203, EAPB0503 has a more pronounced inhibitory activity on OCI-AML3 and IMS-M2 cells as well as on cells derived from *NPM-1* mutant AML patients. Introducing mutated NPM-1 into cells harboring *wt-NPM-1* sensitizes them to EAPB0503. EAPB0503 induced massive apoptosis as demonstrated with the cell cycle analysis by the accumulation of treated cells in the preG0 region. Apoptosis has been confirmed by Annexin V positivity, PARP cleavage, and dissipation of mitochondrial membrane potential in treated OCI-AML3 cells. Moreover, EAPB0503 increased the expression and phosphorylation levels of p53 as well as the upregulation of the p53 downstream effector p21.

We demonstrated that the EAPB0503 induces proteasome-mediated degradation of mutant NPM-1, and restoration of the nucleolar localization of the NPM-1wt leading to an inhibition of the proliferation of *NPM-1* mutant AML cells. Furthermore, EAPB0503 showed

a reduction of bone marrow leukemia burden selectively in *in vivo* *NPM-1 mutant* AML xenograft animals.

These results in growth inhibition and apoptosis, selectively in AML cells that harbor the *NPM-1* mutation reinforce the idea targeting mutated NPM-1 oncoprotein to eradicate leukemic cells and warrant a broader preclinical then clinical evaluation of this promising drug.

In conclusion, our studies highlight the use of EAPB0503 as a promising anti-tumor activity to be investigated preclinically in AML targeted therapy.

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1. Introduction

1.1. Acute Myeloid Leukemia

Acute myeloid leukemia or AML is a heterogeneous disorder characterized by an abnormal development of myeloblasts in the bone marrow. Being previously incurable, success rate of treatment is now between 35% and 40% in AML patients aged <60 years old (Döhner, Weisdorf et al. 2015). For adults with a median age of >60 years old, AML is the most frequent hematological malignancy with an improving prognosis that remains frustrating. It has been shown, very recently, that the disorder results from a series of repeated and cumulative alterations in the genetic of hematopoietic stem cell (Saultz and Garzon 2016). Even with the better understanding of AML biology, all efforts toward changing its treatment strategy have been unsatisfactory (Saultz and Garzon 2016).

1.1.1. Genetics and Classification of AML

AML has been classified, over the years, based on morphology, etiology, genetics and immune-phenotype. Before forty years, the French-American-British (FAB) classification system of AML came out with 8 main AML subtypes, according the following criteria:

- Morphology
- Immune-phenotype/cyto-chemistry (Bennett, Catovsky et al. 1976) (Table 1).

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

FAB Subtype	Name	Adult AML patient (%)
M0	Undifferentiated acute myeloblastic leukemia	5
M1	Acute myeloblastic leukemia with minimal maturation	15
M2	Acute myeloblastic leukemia with maturation	25
M3	Acute promyelocytic leukemia	10
M4	Acute myelomonocytic leukemia	20
M4eos	Acute myelomonocytic leukemia with eosinophilia	5
M5	Acute monocytic leukemia	10
M6	Acute erythroid leukemia	5
M7	Acute megakaryocytic leukemia	5

However, the World Health Organization (WHO) revealed and published a newer classification of AML. This new classification became the today's essential modality for AML.

In 2008, The WHO classification was updated as per the following (Vardiman, Thiele et al. 2009) (Table 2):

Table 2. WHO classification of acute myeloid leukemia.

AML with recurrent genetic Abnormalities	<ul style="list-style-type: none"> • AML with t(8;21)(q22;q22) • AML with abnormal BM* eosinophils and inv(16)(p13q22) or t(16;16)(p13;q22) • Acute promyelocytic leukemia with (15;17)(q22;q12), (PML-RARα)* and variants • AML with 11q23 (MLL)* abnormalities
AML with multilineage dysplasia	<ul style="list-style-type: none"> • Following MDS* or MDS/MPD* • Without antecedent MDS or MDS/MPD, but with dysplasia in at least 50% of the cells
AML and MDS, therapy related	<ul style="list-style-type: none"> • Alkylating agent/radiation-related type • Topoisomerase II inhibitor-related type • Others
AML, not otherwise categorized	<ul style="list-style-type: none"> • AML minimally differentiated • AML without maturation • AML with maturation • Acute myelomonocytic leukemia • Acute monoblastic leukemia • Acute erythroid leukemia • Acute basophilic leukemia • Myeloid sarcoma • Acute panmyelosis

*BM: Bone marrow; MDS: Myelodysplastic syndromes; MPD: Myeloproliferative disorders; PML-RAR α : promyelocytic leukemia-retinoic acid receptor alpha; MLL: Mixed-lineage leukemia

However, a very recent study has proposed a new classification of AML according to many additional leukemia genes and driver mutations (Papaemmanuil, Gerstung et al. 2016) (Table 3).

Table 3. Newly proposed genomic classification of acute myeloid leukemia.

Genomic Subgroup	Frequency in the Study Cohort (N=1540) no. of patients (%)	Most Frequently Mutated Genes* gene (%)
AML with <i>NPM-1</i> mutation	418 (27)	<i>NPM-1</i> (100), <i>DNMT3A</i> (54), <i>FLT3^{ITD}</i> (39), <i>NRAS</i> (19), <i>TET2</i> (16), <i>PTPN11</i> (15)
AML with mutated chromatin, RNA-splicing genes, or both†	275 (18)	<i>RUNX1</i> (39), <i>MLL^{PTD}</i> (25), <i>SRSF2</i> (22), <i>DNMT3A</i> (20), <i>ASXL1</i> (17), <i>STAG2</i> (16), <i>NRAS</i> (16), <i>TET2</i> (15), <i>FLT3^{ITD}</i> (15)
AML with <i>TP53</i> mutations, chromosomal aneuploidy, or both ‡	199 (13)	Complex karyotype (68), -5/5q (47), -7 /7q (44), <i>TP53</i> (44), -17 /17p (31), -12/12p (17), +8/8q (16)
AML with <i>inv(16)</i> (p13.lq22) or <i>t(16;16)</i>(p13.l;q22);<i>CBFB-MYH11</i>	81 (5)	<i>inv(16)</i> (100), <i>NRAS</i> (53), +8/8q (16), +22 (16), <i>KIT</i> (15). <i>FLT3^{TKD}</i> (15)
AML with biallelic <i>CEBPA</i> mutations	66 (4)	<i>CEBPA^{biallelic}</i> (100), <i>NRAS</i> (30), <i>WT1</i> (21), <i>GATA2</i> (20)
AML with <i>t(15;17)</i>(q22;q12); <i>PML-RARA</i>	60 (4)	<i>t(15;17)</i> (100), <i>FLT3^{ITD}</i> (35), <i>WT1</i> (17)
AML with <i>t(8;21)</i> (q22;q22); <i>RUNX1-RUNX1T1</i>	60 (4)	<i>t(8;21)</i> (100). <i>KIT</i> (38), -Y (33), -9q (18)
AML with <i>MLL</i> fusion genes; <i>t(x;l)(x;q23)</i>§	44 (3)	<i>t(x;lq23)</i> (100). <i>NRAS</i> (23)
AML with <i>inv(3)</i> (q21q26.2) or <i>t(3;3)</i> (q21;q26.2); <i>GATA2</i>, <i>MECOM(EV11)</i>	20 (1)	<i>inv(3)</i> (100), -7 (85), <i>KRAS</i> (30), <i>NRAS</i> (30), <i>PTPN11</i> (30), <i>ETV6</i> (15), <i>PHF6</i> (15), <i>SF3B1</i> (15)
AML with <i>IDH2^{R172}</i> mutations and no other class-defining lesions	18 (1)	<i>IDH2^{R172}</i> (100), <i>DNMT3A</i> (67), +8/8q (17)
AML with <i>t(6;9)</i> (p23;q34); <i>DEK-NUP214</i>	15 (1)	<i>t(6;9)</i> (100), <i>FLT3^{ITD}</i> (80), <i>KRAS</i> (20)
AML with driver mutations but no detected class-defining lesions	166 (11)	<i>FLT3^{ITD}</i> (39), <i>DNMT3A</i> (16)
AML with no detected driver mutations	62 (4)	
AML meeting criteria for ≥2 genomic subgroups	56 (4)	

* Genes with a frequency ≥15% with decreasing order of frequency. In bold are the key contributing genes in each class.

† Classification requires one or more driver mutations in *RUNX1*, *ASXL1*, *BCOR*, *STAG2*, *EZH2*, *SRSF2*, *SF3B1*, *U2AF1*, *ZRSR2*, or *MLL^{PTD}*. In the presence of other class-defining lesions --- namely, *inv(16)*, *t(15;17)*, *t(8;21)*, *t(6;9)*, *MLL* fusion genes, or complex karyotype or driver mutations in *TP53*, *NPM-1*, or *CEBPA^{biallelic}* --- two or more chromatin-spliceosome mutations are required.

‡ Classification requires *TP53* mutation, complex karyotype, or in the absence of other class-defining lesions, one or more of the following: -7/7q, -5/5q, -4/4q, -9q, -12/12p, -17/-17p, -18/18q, -20/20q, +11/11q, +13, +21, or +22.

§ Multiple fusion partners for *MLL* were found, with the clinical implications depending on the specific fusion partner.

Based on set of causes, AML can also be subdivided into 3 different categories (Lindsley, Mar et al. 2015):

(1) Secondary AML which is linked to myelodysplastic syndrome (MDS) or disorder

(2) Therapy-related AML which is linked to previous exposure to chemotherapy

(3) *De novo* AML

In contrast, between 40% and 50% of all AML cases are cytogenetically normal (CN-AML) when assessed using conventional banding analysis (Gaidzik and Döhner 2008). Despite the intermediate relapse risk in this category, clinical outcome shows a significant diversity in this population. Treatment strategy and prognostic categorization critically require good molecular screening of AML (Saultz and Garzon 2016).

1.1.2. Clinical manifestations of AML

1.1.2.1. Symptoms and diagnosis

Either treated or untreated, the clinical course of AML is complex. Many complications are commonly seen among symptoms and signs in AML as anemia, thrombocytopenia, leukopenia (Bacârea 2012).

With no direct link with the severity of anemia, AML patients experience other symptoms including: fatigue, bruising, weakness, anxiety, dizziness, depression, bleeding, appetite loss, shortness of breath, and general lack of wellness (Tomaszewski, Fickley et al. 2015). Uncommonly, bleeding in other organs may happen such as stomach, intestine, genital-urinary, lung or central nervous system (Bacârea 2012).

According to Hu et al. the frequencies of the most important presenting features in AML are presented below in (Table 4) (Hu et al., 2011).

Table 4. The frequencies of the most important presenting features in AML, according to Hu et al.

Acute Myeloid Leukaemia		
Initial symptom	Younger Group (age < 60) %	Elderly Group (age ≥ 60) %
Hypodynamia	57.8	68.3
Pale face	60.3	55.5
Fever	40.5	33.5
Dizziness	14.7	10.4
Hemorrhagic dermatologic mucosa	15.5	14.0
WBC infiltration	6.9	12.2
Myalgias	4.3	3.0
White cell count (10⁹/L)		
< 4	24.1	40.2
4 – 10	16.4	9.8
> 10	59.5	50.0
Hemoglobin (g/L)²		
Abnormal	93.1	95.7
Normal	6.9	4.3
Platelets (10⁹/L)		
≤10	29.3	21.3
>10	70.7	78.7
Bone marrow cellularity		
Severely hypercellular	53.4	30.5
Moderately hypercellular	21.6	34.8
Normocellular	13.8	20.7
Hypocellular	11.2	14.0

1.1.2.2. Age

In AML, age is not the only significant factor affecting treatment outcomes. Cytogenetic studies showed that the percentage of patients with favorable cytogenetics decreases dramatically as age increases (≥ 65 years old), and contrariwise, the percentage of those with unfavorable risk cytogenetics remarkably increased (Appelbaum, Gundacker et al. 2006). However, long-term survivors were found among elderly when given intensive treatment. Therefore, elderly AML patients whose age up to 80 years old should be considered fit for intensive therapy (Juliussen, Antunovic et al. 2009).

1.1.3. Treatment strategies

Putting aside the molecular abnormalities, the two most vital factors for AML prognosis are: age and performance status that quantifies general well-being and daily-life activities of the patient (Saultz and Garzon 2016).

In 2010, an effort was made by the European LeukemiaNet (ELN) classification system in order to standardize the prediction and the identification of risk levels in adult AML patients by including cytogenetic and identified molecular alterations (Döhner, Estey et al. 2010). Accordingly, patients are classified into one of four risk groups (Table 5) (Döhner, Estey et al. 2010, Mrózek, Marcucci et al. 2012):

Patients who have monosomal karyotype are those who have at least one monosomy. These patients present a very poor prognosis (the survival rate at 4 years is $< 4\%$) (Medeiros, Othus et al. 2010). Studies revealed that the age of elderly patients can predict alone their poor outcomes irrespective to the classification made by the ELN (Mrózek, Marcucci et al. 2012).

Table 5. Current Stratification of Molecular Genetic and Cytogenetic Alterations, According to ELN Recommendations.

Risk Group	Subsets
Favorable	t(8;21)(q22;q22); RUNX1-RUNX1T1 inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11 Mutated <i>NPM-1</i> without FLT3-ITD (normal karyotype) Biallelic mutated CEBPA (normal karyotype)
Intermediate-I	Mutated <i>NPM-1</i> and FLT3-ITD (normal karyotype) Wild-type <i>NPM-1</i> and FLT3-ITD (normal karyotype) Wild-type <i>NPM-1</i> without FLT3-ITD (normal karyotype)
Intermediate-II	t(9;11)(p22;q23); MLLT3-KMT2A Cytogenetic abnormalities not classified as favorable or adverse
Adverse	inv(3)(q21q26.2) or t(3;3)(q21;q26.2); GATA2-MECO (EVI1) t(6;9)(p23;q34); DEK-NUP214 t(v;11)(v;q23); KMT2A rearranged -5 or del(5q); -7; abnl(17p); complex karyotype *

Abbreviations: ITD, internal tandem duplication; * A complex karyotype is defined as three or more chromosome abnormalities; in the absence of one of the WHO designated recurring translocations; or inversions: t(8;21), inv(16) or t(16;16), t(15;17), t(9;11), t(v;11)(v;q23), t(6;9), inv(3) or t(3;3).

1.1.3.1 Standard therapy

Most types of AML and in most cases have a typical treatment that starts with chemotherapy.

This treatment needs to start immediately after the diagnosis and is usually divided into 2 phases:

- Remission induction (often called induction)
- Consolidation (post-remission therapy)

1.1.3.1.1 Induction therapy

The ultimate goal of induction chemotherapy remains to achieve a morphologic Complete Remission (CR). For the last 40 years, the backbone of intensive induction chemotherapy remained unchanged for both adults whose age is below 60 and old patients having more than 60 years old (Saultz and Garzon 2016). This standard treatment consists of a combination of cytarabine with an anthracycline, and is called the “7 + 3” induction therapy regimen. The typical dose and schedule include either idarubicin (10–12 mg/m² on days 1, 2 and 3) or daunorubicin (60 or 90 mg/m² on days 1, 2 and 3) given with 7 days of nonstop cytarabine infusion (daily dose of 100 mg/m² for 1 week) (Saultz and Garzon 2016) (Table 5).

Using this standard treatment regimen, young, *de novo*, AML patients achieve CR rate between 65% and 73% while only 38% to 62% of elderly AML patients (age > 60 years) achieve CR (Estey and Döhner 2006, Fernandez, Sun et al. 2009). Several trials have now shown that higher dose of anthracycline (90 instead of 45mg/m²) increases the duration of overall survival (OS) and leads to higher CR rates in both young and adults (from 60 to 65 years old) (Fernandez, Sun et al. 2009, Löwenberg, Ossenkoppele et al. 2009).

Moreover, for patients having a *FLT3* mutation, FLT3 inhibitors represent the best treatment agent. In this case, it is necessary to add midostaurin, to standard induction therapy (Stone, O'Donnell et al. 2004). In older adults who have complex karyotype and non-mutated *NPM-1*, the use of hypomethylating agents (like decitabine and azacitidine) have shown good results (Blum, Garzon et al. 2010, Klepin 2014).

1.1.3.1.2. Consolidation therapy

In order to avoid relapse and eliminate Minimal Residual Disease (MRD) in the bone marrow after induction, post-induction or consolidation therapy is given as a stepping stone to transplant or to achieve cure.

Assessment of MRD using real-time PCR or Next Generation Sequencing (NGS) is progressively being used to help track treatment response and in predicting future relapse. This assessment has been shown to be superior than morphology alone (Grimwade and Freeman 2014, Kohlmann, Nadarajah et al. 2014). Despite this very helpful information, the heterogeneity of AML made it very hard for the following mutational clones to know the risk of leukemia expansion, because some clones can persist in patients in long-term remission after the treatment, such as DNMT3A mutation. This mutation has been detected lately in AML evolution and expanded the pre-leukaemic pool of hematopoietic stem cells from which AML evolves (Shlush, Zandi et al. 2014).

In general, there are two main strategies for consolidation (Estey and Döhner 2006, Schlenk 2014)

- Chemotherapy (including targeted agents)
- Hematopoietic stem cell transplantation

Depending on the availability of a stem cell donor, the type of leukemia and the fitness of the patient, these two main strategies could be used independently or in combination for the majority of cases.

Trying to establish a regimen that prolongs remission and improves survival in young adults with favorable risk (<60 year of age), an intermediate-dose of cytarabine 1.5 g/m² twice daily on days 1, 3 and 5 is given in three to four cycles, and seems to be effective (Byrd, Mrózek et al. 2002). Bone marrow transplantation is reserved for these patients only at

relapse (Estey and Döhner 2006). For these adults, a cytarabine dose of 3 g/m², for three courses, gave the same results, as compared to a lower dose of 1.5 g/m² (Burnett, Russell et al. 2013) (Table 6). Therefore, this lower dose remained the standard of treatment.

For patients with intermediate or high risk disease and after reaching CR, the most effective long term therapy is the allogeneic Stem Cell Transplantation (allo-SCT) (Appelbaum 2003, Popat, de Lima et al. 2012). Even though, lack of suitable donor and other factors make many patients not eligible for transplantation (Appelbaum 2003).

Table 6. AML induction and consolidation therapies (O'Donnell 2014).

Induction		Consolidation	
AML			
Ara-C	200 mg/m ² IV as continuous infusion x 7 d	Ara-C ^b	3 g/m ² q12h IV as 2- to 3-h infusion on days 1, 3, and 5; repeat q28d x 4 cycles
IDA ^a	12 mg/m ² IV on days 1-3		
Or			
Daun	60-90 mg/m ² on days 1-3		
ALSG regimen			
Ara-C ^b	3 g/m ² IV q12h as 2- to 3-h infusion on days 1, 3, 5, and 7 (8 doses)	Ara-C	100 mg/m ² IV as continuous infusion x 5 d
Daun	45-60 mg/m ² IV on days 1-3	Daun	50 mg/m ² IV x 2 d 75
VP-16	75 mg/m ² IV x 7 d	VP-16	mg/m ² IV x 5 d

ALSG = Australian Leukemia Study Group; AML = acute myelogenous leukemia;

Ara-C = cytarabine; Daun= daunorubicin; IDA= idarubicin; VP-16 = etoposide

^a Idarubicin has been substituted for daunorubicin, 45 mg/m², which had been the prevalent anthracycline used in clinical trials prior to 1993. Mitoxantrone, 10 mg/m² x 5 days, has also been used as an alternative.

^b For patients < 60 years of age

1.1.3.1.3. Maintenance therapy

Only a small fraction of the patients who relapse, reach a successful second remission using salvage chemotherapy followed by stem cell transplantation (Estey and Döhner 2006). Salvage regimes include high doses of cytarabine (2 to 3 g/m² for 8 to 12 doses) paired with mitoxantrone, etoposide, methotrexate, or fludarabine (Table 7), or intermediate dose of cytarabine (between 500 and 1500 mg/m² given intravenously every 12 hours on days 1 to 3); Mitoxantrone (MEC) at the dose of 8 mg/m², Etoposide at the dose of 100 mg/m², and Cytarabine at the dose of 100 mg/m² on days 1 to 5) or Fludarabine (FLAG-IDA) at the dose of 30 mg/m² given intravenously on days 1 to 5 (or a dose of 20 mg/m² for elderly patient aged over 60 years old), or lastly Cytarabine at the dose of 1500 mg/m² (or a dose between 500 and 1000 mg/m² in elderly patient aged over 60 years old) given intravenously, followed 4 hours later with fludarabine infusion, on days 1 through 5; Idarubicin dose of 8 mg/m², given intravenously, on days 3 through 5; Granulocyte colony-stimulating factor 5 µg/kg, subcutaneously, from day 6 to white-cell count >1 g/L (FLAG-IDA) (Döhner, Weisdorf et al. 2015). The likelihood of achieving a second complete remission is best in younger age patients with a long first remission, and in those with favorable cytogenetics (Breems, Van Putten et al. 2005).

Table 7. AML relapse therapy (O'Donnell 2014).

Ara-C	2-3 g/m ² IV q12h as 3-h infusion x 8 doses	plus	Mitox	12 mg/m ² IV on days 1-3 ^a
				Or
			Daun	60 mg/m ² IV on days 5 and 6
				Or
			VP-16	100 mg/m ² IV daily x 5 d ^a
Ara-C	2 g/m ² /d IV x 5 d	plus	FdURD	300 mg/m ² /d x 5 d + G-CSF ± Ida 10 mg/m ² /day on days 1-3
Mitox	10 mg/m ² IV	plus	VP-16	100 mg/m ² IV as 2-h infusion daily x 5 d

AML = acute myelogenous leukemia; Ara-C = cytarabine; Daun = daunorubicin;
 FdURD = fludarabine; G-CSF = granulocyte colony-stimulating factor; Ida = idarubicin;
 Mitox = mitoxantrone; VP-16 = etoposide

^a Also used for relapsed acute lymphoblastic leukemia

1.1.3.2. High dose daunorubicin

Up to 80% of young adults and to 60% of older adults can achieve a CR with the standard induction regimen that consists of 45 mg/m² of daunorubicin (DNR) intravenously for 3 days and 100 mg/m² of cytarabine (Ara-C) by continuous infusion for 7 days (Dillman, Davis et al. 1991). Several major studies have shown that higher doses of DNR (80 or 90 mg/m²) can be administered safely (Kolitz, George et al. 2004, Pautas, Merabet et al. 2010). A high dose of DNR 90 mg/m² was compared versus 45 mg/m² in patients aged older than 60 years by the Dutch-Belgium Hemato-Oncology Cooperative Group (HOVON)/Swiss Group for Clinical Cancer Research (SAKK) (Löwenberg, Ossenkoppele et al. 2009).

Although the exact optimal dosage of DNR is not yet established, trials conclude that DNR dose should be between 60 mg/m² to 90 mg/m² for 3 days (Rowe 2009).

A recent study showed that there is no benefit of very high doses of DNR (for example 90 mg/m² over 60 mg/m²). In addition to many concerns about toxicity of high-dose DNR, the wide use of the 60-mg/m² dose is adopted as a newer “standard”. These led National Cancer Research Council (NCRC) the United Kingdom to conduct a trial to compare DNR at 60 with 90 mg/m² in the induction of a group of AML patients (Burnett, Russell et al. 2015). The findings revealed that the cumulative dose of anthracyclines in a dose of 60 mg/m² was almost the same as the higher dose of 90 mg/m². A lower dose of 45 mg/m² seems insufficient (Fernandez 2015). Thus, it is clear that a DNR dose 60 mg/m² is sufficient and better with less risk of toxicity.

1.1.3.3. New formulations of old agents

Toxicity and high drug doses can be reduced by liposomal encapsulation of drugs. CPX-351 is a liposomal formulation that encapsulates cytarabine and DNR at a 5:1 molar ratio (El-Cheikh and Crocchiolo 2012).

A completed phase 1 study (Feldman, Lancet et al. 2008) showed that liposomal encapsulation of this chemotherapy changed the safety profile by reducing non-hematologic, gastrointestinal and hepatic toxicities while retaining hematopoietic cytotoxicity.

1.1.3.4. FLT3 inhibitors

In 30% of all AML patients, The *Flt3*-Internal Tandem Duplication (ITD) confers a poor risk status characterized by an increased relapse rate and poor overall survival (Gregory, Wald et al. 2009, Kayser, Schlenk et al. 2009).

Abnormal activation of FLT3-kinase is considered to be a good target in AML therapy. Many chemical compounds have been synthesized and tested as FLT3-tyrosine kinase inhibitors (TKIs) and examined in AML patients in single or in combination with chemotherapy. These include Midostaurin (PKC412), Lestaurtinib (CEP-701), Semaxanib (SU5416), Sorafenib (BAY 43-9006) and others... (Kindler, Lipka et al. 2010). Sorafenib is a multikinase inhibitor that is approved for the treatment of metastatic renal cell carcinoma (Kane, Farrell et al. 2006) and has a significant clinical activity in *Flt3-ITD* positive AML (Metzelder, Wang et al. 2009, Schroeder, Saure et al. 2009).

In patients with FLT3 mutations, using a combination therapy of Idarubicin and Cytarabine with sorafenib seems effective since mutant clones were reduced but not completely eliminated (Al-Kali, Jones et al. 2009). Research results and data suggest integrating sorafenib into induction and consolidation regimens to achieve maximal outcome (El-Cheikh and Crocchiolo 2012).

1.1.3.5. Other agents in clinical development

Researchers at medical centers, universities and other institutions are always trying to improve the treatment of AML. Many studies on several agents are being done in order to find more effective and safer treatments for AML (Table 8).

Table 8. Agents in clinical trial.

Compound	Highlights	Mode of action	Ref.
<i>Voreloxin</i>	A naphthyridine analogue	<ul style="list-style-type: none"> • Intercalates DNA • Inhibits topoisomerase II • Induces apoptosis 	Ravandi, Kantarjian et al. 2009
<i>Amonafide L-malate (AS1413)</i>	An imide derivative of naphthalic acid used in combination with cytarabine	<ul style="list-style-type: none"> • Intercalates DNA • Produced a high complete remission rate and durable responses in patients with secondary AML 	Erba, O'Donnell et al. 2009
<i>Lenalidomide</i>	An imide derivative of naphthalic acid used in combination with cytarabine and daunorubicin in high risk MDS/AML with del 5q	<ul style="list-style-type: none"> • Intercalates DNA • Inhibits topoisomerase II • Produced response rate of 28% without significant additional toxicity 	Zhu, Ma et al. 2010
<i>Filanesib (ARRY-520)</i>	<ul style="list-style-type: none"> • Potent inhibitor of the kinesin spindle protein (KSP) • Required for cell cycle progression through mitosis 	<ul style="list-style-type: none"> • Induction of cell cycle arrest 	Garcia-Manero, Khoury et al. 2009
<i>Barasertib AZD1152</i>	Dihydrogen phosphate prodrug of the pyrazoloquinazoline Aurora kinase inhibitor	<ul style="list-style-type: none"> • Inhibits human tumor xenograft growth • Induces apoptosis • Inhibits tumor growth in vivo 	Wilkinson, Odedra et al. 2007 Lowenberg, Rousselot et al. 2009
<i>Selumetinib (AZD6244)</i>	An orally small molecule with antineoplastic activity	Inhibits MEK kinase	Frémin and Meloche 2010, Zhu, Ma et al. 2010
<i>Terameprocol</i>	A synthetic tetra-methylated derivative of nordihydroguaiaretic acid (NDGA)	Inhibits the production and activation of surviving	Grossman, Ye et al. 2012
<i>Azacitidine</i>	A pyrimidine nucleoside analogue of cytidine	Inhibits DNA methyltransferase	Estey 2013

1.1.3.6. Allogeneic Stem Cell Transplant (allo-SCT)

To start the work-up process for allo-SCT in patients with AML, published guidelines and treatment recommendations (regarding disease-risk, age, comorbidities choice of donor and others...) are usually the basis (Ljungman, Bregni et al. 2010). For AML patients, the use of myeloablative and nonmyeloablative allogeneic stem cell transplantation represents a potentially curative approach (El-Cheikh and Crocchiolo 2012). Knowing the increase in need for SCT, the challenges remain in improving the transplant related activities from the choice of donor, the availability of another donors, the preparative regimen and preventive measures (Aljurf, Nassar et al. 2015).

However, it is well known that benefits from allogeneic SCT are not guaranteed for all AML patients. Therefore, establishing definitive and clear recommendations to benefit from transplant is needed.

1.2. Nucleophosmin (NPM-1)

1.2.1. Introduction

Nucleophosmin 1 (NPM-1, also referred to as B23) was originally identified as a phosphoprotein and is a member of the nucleoplasmin family of nuclear chaperons [NPM-1, NPM-2 and NPM-3] (Mamrack, Olson et al. 1979, Chang, Dumbar et al. 1988, Frehlick, Eirín - López et al. 2007) (Table 9).

Table 9. NPM family members.

Member	Also known as	Location
nucleoplasmin member 1 (NPM-1)	B23 or numatrin in mammals and NO38 in amphibians	Mainly nucleolar; wide tissue distribution
nucleoplasmin member 2 (NPM-2)	Nucleoplasmin in amphibians	Nuclear; only found in eggs and oocytes
nucleoplasmin member 3 (NPM-3)	NO29 in amphibians	Mainly nucleolar; wide tissue distribution

NPM-1 was referred to as B23 because it was the 23rd protein in region B on the slab-gel electrophoresis where the numbering of protein spots was in increasing order in term of mobility in the two-dimensional polyacrylamide gel (Orrick, Olson et al. 1973). NPM-1 is a nuclear and cytoplasmic shuttling protein and its continuous shuttling is critical for many biological functions (Grisendi, Mecucci et al. 2006, Naoe, Suzuki et al. 2006, Meani and Alcalay 2009).

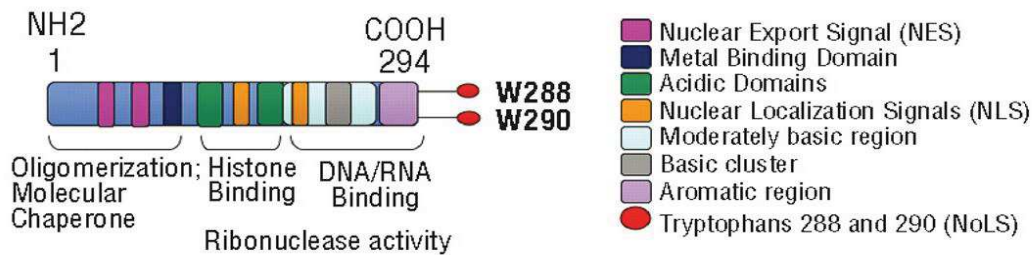
NPM-1 has many roles in cell growth and proliferation due to its engagement in many biological processes, including maintenance of genomic stability, centrosome duplication, ribosome biogenesis, DNA repair, inhibition of apoptosis, chaperoning of histones, regulation of cell cycle, and regulation of the ARF-p53 tumor suppressor pathway (Okuda 2002, Bertwistle, Sugimoto et al. 2004, Kurki, Peltonen et al. 2004, Swaminathan, Kishore et al. 2005, Yu, Maggi et al. 2006, Murano, Okuwaki et al. 2008, Falini, Gionfriddo et al. 2011, Falini, Martelli et al. 2011, Federici and Falini 2013).

Starting from the N-terminus, NPM-1 shares a conserved core region (oligomerization domain) with other family proteins of NPM (Herrera, Correia et al. 1996) that is required for protein oligomerization (Okuwaki 2008) and plays an important role in ribosomal biogenesis and p53-mediated tumor suppression (Murano, Okuwaki et al. 2008, Colombo, Alcalay et al. 2011).

Another domain in NPM-1, enriched with lysine and arginine (the basic domain), is important for nucleic acid binding (Hingorani, Szebeni et al. 2000) that may explain the role of NPM-1 in nucleosome assembly and ribosomal biogenesis in cells.

In addition to subcellular localization and binding partners of NPM-1, its post-translational modifications dictate its multifunctional characteristic (Ramsamooj, Notario et al. 1995, Tokuyama, Horn et al. 2001, Sato, Hayami et al. 2004, Liu, Liu et al. 2007, Shandilya, Swaminathan et al. 2009). The multifunctional ability of NPM-1 is due to its structure that contains distinct structural domains (Figure 1) (Hingorani, Szebeni et al. 2000, Okuwaki 2008, Qi, Shakalya et al. 2008, Yip, Siu et al. 2011).

Figure 1. Structure and functional domains of wild-type NPM-1 (Falini, Nicoletti et al. 2007).



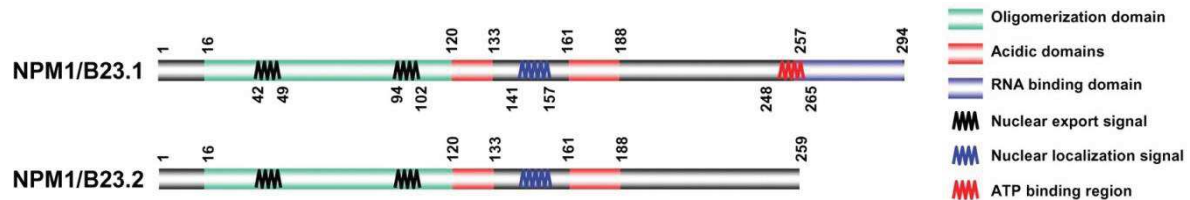
Beginning with the N-terminus, the protein shows two nuclear export signal (NES) motifs (residues 42–49 and 94–102), a metal binding domain, two acidic regions (residues 120–132 and 160–188), a two-part nuclear localization signal (NLS) motif (residues 152–157 and 190–197), a basic cluster inside a basic region, and an aromatic region at the C-terminus unique to *NPM-1* that contains the nucleolar localization signal (NLS) with tryptophan residues 288 and 290 (Falini, Nicoletti et al. 2007).

1.2.2. Structural and Functional Domains of NPM-1

The *NPM-1* gene is located on chromosome 5q35 and consists of about 23 kb that form 12 exons (Chang and Olson 1990). It has two splicing variants, B23.1 (isoform 1) and B23.2 (isoform 2) (Wang, Umekawa et al. 1992) (Figure 2). Both isoforms are expressed in growing cells in order to form hetero-oligomers, but B23.2 (isoform 2) is localized all over the nucleus

since it does not have the RNA-binding domain, and the balanced expression levels of both isoforms seems to be important for NPM-1 function (Okuwaki 2008).

Figure 2. The two splicing variants of NPM-1 (Okuwaki 2008).



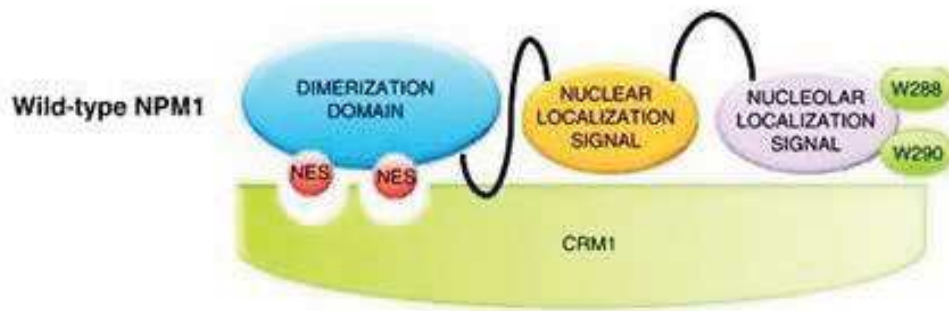
B23.1 is the only protein among the NPM-1 family that has the RNA-binding domain at its C-terminus.

NPM-1 is expressed in all the tissues but mainly localized in the nucleolus. The biological and physiological implications of B23.2 are still unclear (Colombo, Alcalay et al. 2011). In addition to its distinct structural domains that have distinct functional properties (Hingorani, Szebeni et al. 2000, Okuwaki 2008, Qi, Shakalya et al. 2008, Yip, Siu et al. 2011), NPM-1 also contain several important, specific sequence motifs that are required for protein transportation (Grisendi, Mecucci et al. 2006, Okuwaki 2008, Falini, Maciejewski et al. 2010). These include:

- Nuclear Export Signal (NES)
- Nuclear Localization Signal (NLS)
- Nucleolar Localization Signal (NoLS)

The NES motifs facilitate the nuclear export NPM-1 by binding to the export receptor CRM1 (also called Exportin 1) (Kau, Way et al. 2004) (Figure 3).

Figure 3. The 3 Functional motifs of Wild-type NPM-1 (NPM-1wt).



Wild-type NPM-1 consists of 3 functional motifs:

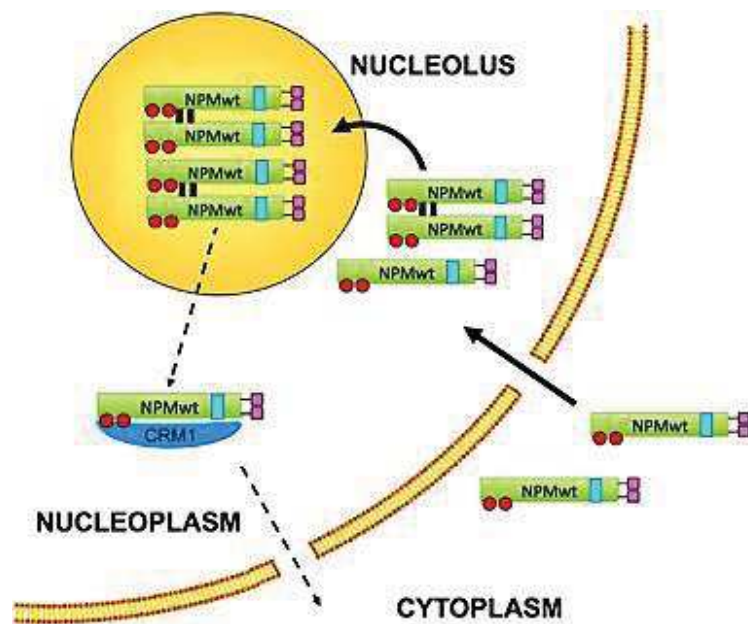
Nuclear export signal (NES) motifs at the N-terminal region (dimerization domain)

Nuclear localization signal (NLS) motif

Nucleolar localization signal (NoLS) motif with 2 tryptophans at positions 288 and 290 at the C-terminal region (Falini, Bolli et al. 2009).

The C-terminal end of NPM-1 is unique to the isoform 1. This region harbors the nucleolar localization signal (NoLS). The two N-terminal NES motifs and C-terminal NoLS motif of NPM-1 play a major role in regulating the shuttling of NPM-1 across the nucleolus, nucleoplasm and the cytoplasm (Federici and Falini 2013) (Figure 4).

Figure 4. Nucleophosmin traffic between the cytoplasm, nucleoplasm and nucleolus

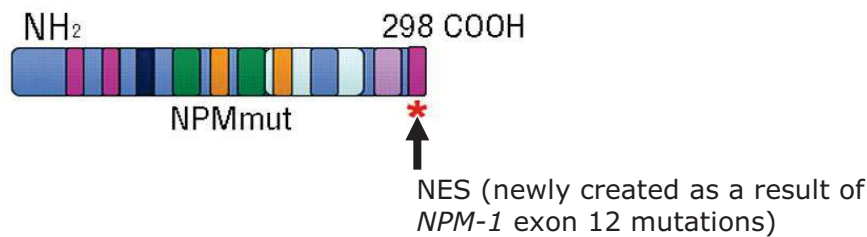


Mechanism of shuttling of wild-type NPM-1wt between the nucleolus and the cytoplasm shows predominance of the nuclear import (arrow) over the nuclear export (dotted arrow) making the wild-type NPM-1 mainly resides in the nucleolus (Falini, Bolli et al. 2009).

1.2.3. *NPM-1* mutations and gene alterations

NPM-1 mutations, in exon 12, occur in about 30% of adult AML patients (female predominance) which make it the most frequent mutation in AML (Schnittger, Schoch et al. 2005, Falini, Nicoletti et al. 2007) (Figure 5).

Figure 5. Structural and functional domains of NPM-1 altered proteins in AML.



Mutated NPM-1 protein in AML: mutations at the NPM-1 C-terminus introduce a new NES motif at the nucleolar binding domain (asterix) (Falini et al. 2007).

Among these mutations, a mutation in exon 12 in the C-terminal domain (CTD), called mutation A, represents the most common mutation in AML (up to 80 % of mutations in the NPM-1 mutated AML patients) and consists of a “TCTG duplication” insertion in the coding sequence. Mutation A leads to an aberrant cytoplasmic localization of NPM-1 and stimulates leukemogenesis in cells as well as in Knock-in and transgenic mice (Falini, Nicoletti et al. 2007, Cheng, Sportoletti et al. 2010, Chou, Ko et al. 2012) (Figure 6). Other types of mutations including type B and type D are less frequent and account together to about 15 % of cases (Thiede, Koch et al. 2006).

Figure 6. Wild-type and mutant sequences of *NPM-1* DNA and their corresponding encoded protein part (Thiede, Koch et al. 2006).

1 2 3 4 5 6 7 8 9 11 12															
DNA												Protein		N	%
CAAGATCTCTG	---	GCAG	---	TGGAGGAAGTCTCTTTAAGAAAATAG	QDLWQWRKSL	wt									
CAAGATCTCTG	CTGG	GCAG	---	TGGAGGAAGTCTCTTTAAGAAAATAG	QDLCCLAVEEVSLRK	Mut A	184		80.3						
CAAGATCTCTG	CATG	GCAG	---	TGGAGGAAGTCTCTTTAAGAAAATAG	QDLCMAVEEVSLRK	Mut B	21		9.2						
CAAGATCTCTG	CTGG	GCAG	---	TGGAGGAAGTCTCTTTAAGAAAATAG	QDLCCLAVEEVSLRK	Mut D	7		3.1						
CAAGATCTCTG	CTGG	GCAG	---	TGGAGGAAGTCTCTTTAAGAAAATAG	QDLCCLAVEEVSLRK	DD-1*	2		0.9						
CAAGATCTCTG	CTGG	GCAG	---	TGGAGGAAGTCTCTTTAAGAAAATAG	QDLCWAVEEVSLRK	DD-2	1		0.4						
CAAGATCTCTG	CAGAG	GCAG	---	TGGAGGAAGTCTCTTTAAGAAAATAG	QDLCRAVEEVSLRK	DD-3	2		0.9						
CAAGATCTCTG	TGTGG	GCAG	---	TGGAGGAAGTCTCTTTAAGAAAATAG	QDLCVAVEEVSLRK	DD-4	1		0.4						
CAAGATCTCTG	TCAGG	GCAG	---	TGGAGGAAGTCTCTTTAAGAAAATAG	QDLCQAVEEVSLRK	DD-5	1		0.4						
CAAGATCTCTG	TATGG	GCAG	---	TGGAGGAAGTCTCTTTAAGAAAATAG	QDLCMAVEEVSLRK	DD-6*	1		0.4						
CAAGATCTCTG	TAAGG	GCAG	---	TGGAGGAAGTCTCTTTAAGAAAATAG	QDLCRAVEEVSLRK	DD-7*	1		0.4						
CAAGATCTCTG	TCGGG	GCAG	---	TGGAGGAAGTCTCTTTAAGAAAATAG	QDLCRAVEEVSLRK	DD-8	1		0.4						
CAAGATCTCTG	---	GCAG AGGA	TGGAGGAAGTCTCTTTAAGAAAATAG	QDLWQRMEEVSLRK	DD-9	2		0.9							
CAAGATCTCTG	---	GCAG AGAA	TGGAGGAAGTCTCTTTAAGAAAATAG	QDLWQRMEEVSLRK	DD-10	1		0.4							
CAAGATCTCTG	---	GCAG AGAC	TGGAGGAAGTCTCTTTAAGAAAATAG	QDLWQRLVEEVSLRK	DD-11	2		0.9							
CAAGATCTCTG	---	GCAG CGCT	TGGAGGAAGTCTCTTTAAGAAAATAG	QDLWQRLVEEVSLRK	DD-12	1		0.4							
CAAGATCTCTG	---	GCAG GGGTGGGAA	TCTCTCTTTAAGAAAATAG	QDLWQGVGNLSLRK	DD-13	1		0.4							

Red font indicates insertion mutations.

Although most NPM-1 is localized mainly to the nucleolus (Andersen, Lam et al. 2005, Lam, Trinkle-Mulcahy et al. 2005), this protein continuously shuttles between the nucleolus, nucleoplasm and cytoplasm of normal cells (Borer, Lehner et al. 1989, Yun, Chew et al. 2003). Under normal conditions, the nuclear import of NPM-1wt predominates over its export, thus explaining why most of the NPM-1wt is found in the nucleolus (Figure 4).

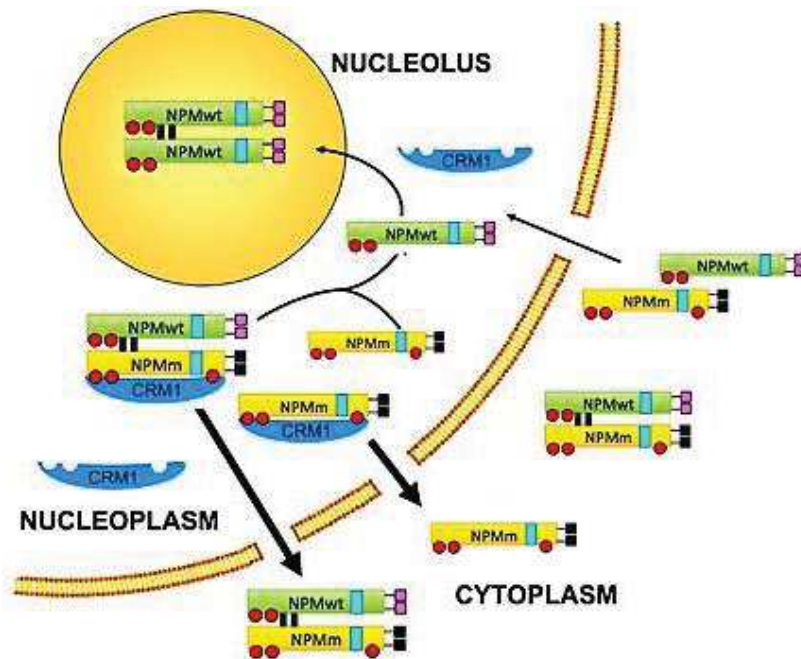
When the mutation occurs, one of the mostly distinctive features of the mutated NPM-1 is its abnormal accumulation, along with its oligomerized wt-NPM-1, in the cytoplasm of leukemic cells (Falini, Mecucci et al. 2005) (Figure 7). This is related to two alterations at C-terminus:

- Generation of an additional Nuclear Export Signal (NES) (Falini, Mecucci et al. 2005)
- Loss of the Nucleolar Localization Signal (NoLS) which is crucial for NPM-1 nucleolar localization (Nishimura, Ohkubo et al. 2002).

Recent studies focused on the localization properties of the wt-NPM-1 and its oligomerization with the mutated NPM-1, to better understand the biology of AML. In the wild-type NPM-1, the C-terminal domain (CTD) has three short helices (H1, H2 and H3) separated by short loops (Grummitt, Townsley et al. 2008). H2 fragment corresponding to the region 264–277 (H2^{264–277}) forms amyloid like aggregates in physiologic conditions and the observation that H2 amyloid like assemblies are cytotoxic suggests that they are active biologic entities. These may be involved in the gain of function observed in NPM-1 mutants (Di Natale, Scognamiglio et al. 2015). Studies showed that both alterations are needed to confirm mutant NPM-1 (NPM-1c) dislocation (Falini, Bolli et al. 2006). More recently, Thioflavin T assays showed that the third helix of the mutated NPM-1-CTD (H3-mutE and the H3-mutA) sequences displayed a significant aggregation propensity and the H3-mutE and H3-mutA peptides, unlike the H3-wt, were moderately and highly toxic, respectively, when exposed to human neuroblastoma cells (Scognamiglio, Di Natale et al. 2016). More importantly, cellular localization experiments confirmed that the mutated sequences hamper their nucleolar accumulation, and that the helical conformation of the H3 region is crucial for such a localization (Scognamiglio, Di Natale et al. 2016).

Clinically, a favorable Overall Survival (OS) is predicted when the mutation is not associated with any other genetic abnormalities (Saultz and Garzon 2016) and the reason behind this improved survival remains unclear (Döhner, Schlenk et al. 2005).

Figure 7. Mechanism of altered nucleocytoplasmic transport of mutant NPM-1 (NPM-1c).



Cytoplasmic over-expression of *NPM-1* (both wild-type and mutated) in AML cell that harbors the mutated *NPM-1* gene. Nuclear export (thick arrows) is much greater than nuclear import (thin arrow).

Purple squares indicate tryptophans 288 and 290

Black squares indicate mutated tryptophans

Turquoise rectangle indicates the nuclear localization signal (NLS) (Falini, Bolli et al. 2009).

1.2.4. Other mutations in AML

NPM-1 mutations can be associated with other recurrent genetic abnormalities such as FMS-like tyrosine kinase 3 (*FLT3-ITD*), *DNMT3A* mutations, *FLT3-TKD* and *IDH* mutations (Marcucci, Haferlach et al. 2011, Marcucci, Metzeler et al. 2012).

The *FLT3* (FMS-like tyrosine kinase 3), discussed earlier, is a Receptor Tyrosine Kinase (RTK) expressed by early myeloid and lymphoid progenitor cells and involved in the proliferation, differentiation, and apoptosis of hematopoietic cells (Stirewalt and Radich 2003, Kindler, Lipka et al. 2010).

Currently, expression of *NPM-1* and *FLT3* mutations is highly considered to decide on therapeutic strategies in AML patients (Wang 2014). *NPM-1* mutations, mainly coexisting

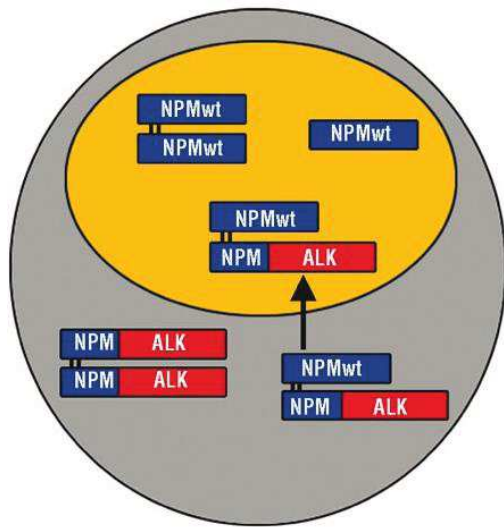
with *FLT3-ITD* mutations, were detected in more than 50% of cases of mutated *NPM-1*, as compared to about 25 % of the cases with the wild-type *NPM-1* (Balusu, Fiskus et al. 2015). The high frequency of coexistence of *FLT3* mutations with mutated *NPM-1* suggests a possible pathogenic link between these two mutations (Balusu, Fiskus et al. 2015).

As per Thiede et al, *NPM-1* mutations are primary events that precede acquisition of *FLT3-ITD* or other mutations. This was based on the fact that *NPM-1* and *FLT3-ITD* mutations can be screened in one multiplex PCR with subsequent fragment analysis were results revealed that *NPM-1* mutations occurred before the *FLT3-ITD* mutations in most cases (Thiede, Koch et al. 2006).

Presence of *FLT3* mutations in patients has been linked with poor prognosis (Gilliland and Griffin 2002) and insertion of *FLT3-ITD* in the tyrosine kinase domain-1 has been associated with bad response to chemotherapy (Kayser, Schlenk et al. 2009).

Other interactions may occur, *NPM-1* gene translocation may take place in lymphoma but rarely in AML (Falini and Mason 2002). However, the interaction with the Anaplastic lymphoma kinase gene *NPM-1-ALK* results may not affect the function the wt-*NPM-1* that retains its nucleolar localization (Falini, Nicoletti et al. 2007) (Figure 8).

Figure 8. Schematic representation of wild-type NPM and NPM-ALK fusion proteins in Anaplastic large cell lymphoma (ALCL).



Cytoplasmic NPM positivity is due to the presence in the cytoplasm of NPM-ALK. Wild-type NPM can form heterodimers with NPM-ALK causing its dislocation into the nucleus (arrow). Wild-type NPM is nucleus-restricted.

1.2.5. Targeted Therapy in AML with Mutated *NPM-1*

The uncontrolled proliferation of malignant cells remains one of the main therapeutic hallmarks of cancer (Hanahan and Weinberg 2011). Therefore, most of the current strategies focus on the inhibition of cell proliferation or the induction of apoptosis in cancer cells. Knowing that NPM-1 plays a major role in the regulation of cell sensitivity, growth and proliferation, overexpression of NPM-1 has been demonstrated to reduce *All-Trans* Retinoic Acid (ATRA)-mediated sensitivity in some leukemic cells (Hsu and Yung 2000) and arrests differentiation (Hsu and Yung 2003).

Downregulation of NPM-1 increases the sensitivity of AML cells to sodium butyrate-induced apoptosis (Liu, Hsu et al. 1999). Recent studies showed that knocking down the total NPM-1 in leukemic cells that harbors the NPM-1c induced p53, p21, and C/EBP- α and depleted HOXA9 and Meis1 mRNA (Balusu, Fiskus et al. 2015). These alterations can morphologically affect the differentiation and reduce the survival of NPM-1c AML cells (Figure 8). Moreover, knocking-down *NPM-1* can considerably sensitize cultured *NPM-1c* AML cells to ATRA, and the anti-leukemic agent Ara-C (Balusu, Fiskus et al. 2011, Qin, Shao et al. 2011). Furthermore, our laboratory has recently demonstrated that arsenic and

ATRA synergistically induce proteasome-mediated degradation of NPM-1c, resulting in differentiation, growth arrest and apoptosis, selectively in AML cells harboring a *NPM-1c* mutation. Moreover, the expression of NPM-1c protein was associated with altered formation of the promyelocytic leukemia protein (PML) nuclear bodies (NBs). The ATRA/arsenic combination significantly reduced leukemic blasts in the bone marrow of three *NPM-1c* AML patients and restored nucleolar localization of NPM-1 and PML NBs both *ex vivo* and *in vivo*. These findings provide a basis for the proposed ATRA-sensitivity in *NPM-1c* AML and warrant a broader evaluation of a therapeutic regimen comprising ATRA/arsenic combination, particularly, in elderly patients (El Hajj, Dassouki et al. 2015). In the context of a back to back parallel acceptance in the same Blood journal volume, an Italian study by Martelli et al., obtained the same results and proposed arsenic and ATRA as prospective drugs for a molecular targeted-based therapy in AML cells that harbor mutant NPM-1 leading to NPM-1c degradation and leukemic cells eradication (Martelli, Gionfriddo et al. 2015) (Figure 9).

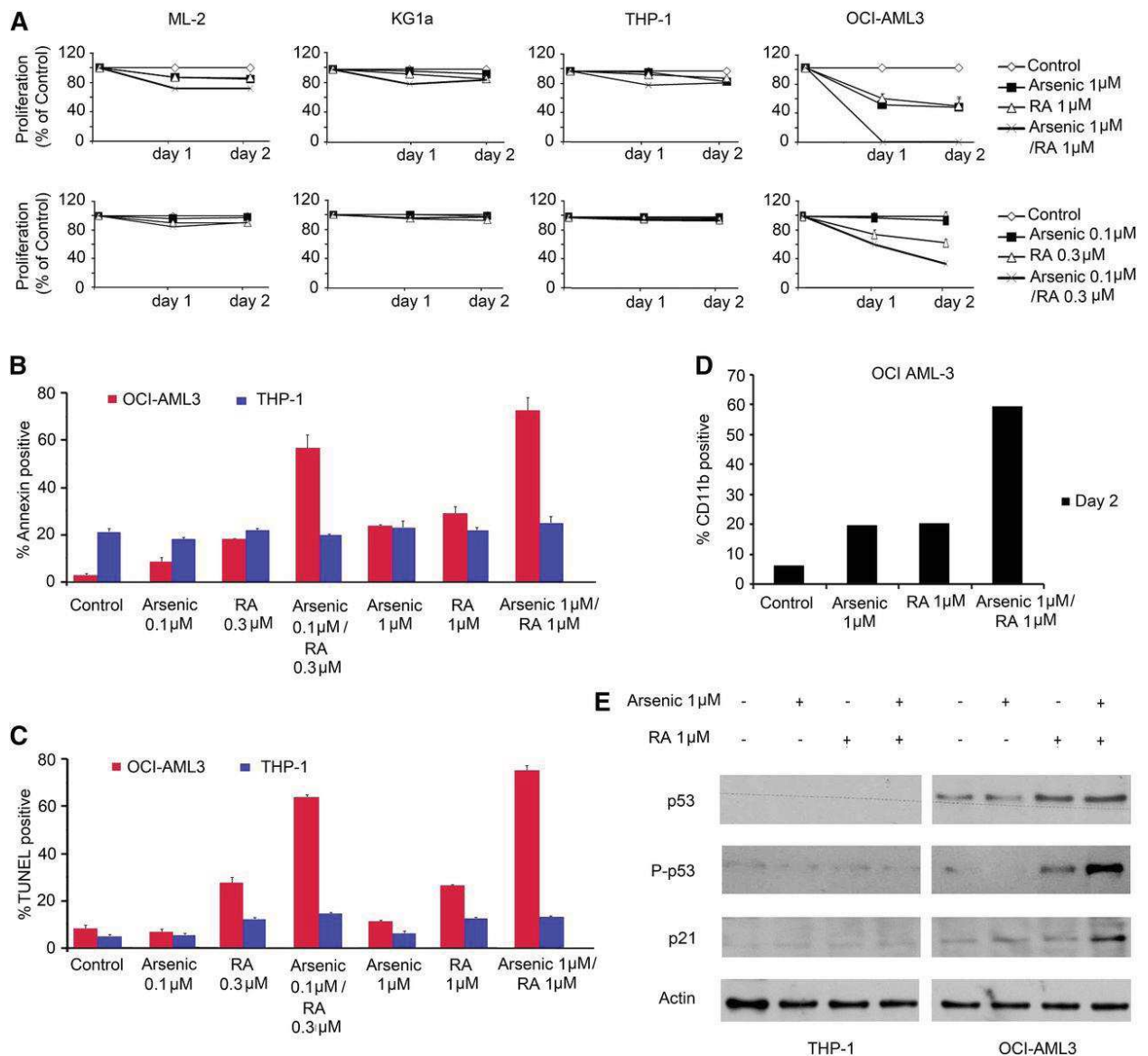
In granulocytic differentiation, studies showed that expression of differentiation marker CD15 decreases (Cho, Yeh et al. 1996, Hsu and Yung 1998), while CD16 would increase (Lund-Johansen and Terstappen 1993). Hsu et al (Hsu and Yung 2000) found that the expression of CD15 decreased in less extent in NPM-1 over-expressed cells (pCR3-B23) as compared with control vector-transfected cells (pCR3) treated with ATRA (10 μ M; 1–3 days). In addition, there was relatively less increase of the expressions of CD16 in PCR3-B23 cells as compared with pCR3 cells treated with ATRA (10 μ M; 5–6 days) (Table 10).

Table 10. Expression of differentiation markers CD15 and CD16 in NPM-1 over-expressed cells (pCR3-B23) and control vector-transfected cells (pCR3) treated with ATRA

	pCR3	pCR3-B23
CD15 expression		
Control	88.82%	80.49%
ATRA, 1 day	56.18%	67.90%
ATRA, 3 days	42.39%	71.56%
CD16 expression		
Control	1.21%	0.69%
ATRA, 5 days	7.92%	2.15%
ATRA, 6 days	17.40%	4.07%

NPM-1 over-expressed (pCR3-B23) or the control vector-transfected (pCR3) HL-60 cells were treated with 10 μ M ATRA for various times (1–3 days or 5–6 days). After the cells were harvested, expressions of the CD15 or CD16 were evaluated by cytofluorimetry using specific anti-CD15 or anti-CD16 FITC-conjugated monoclonal antibodies.

Figure 9. ATRA and Arsenic induce growth inhibition and apoptosis in NPM-1 mutated AML cell line(El Hajj, Dassouki et al. 2015).



A. AML cell lines with normal NPM-1 (ML-2, KG1a, and THP-1) or mutated NPM1 (OCI-AML3 and IMS-M2) were treated with arsenic (1 μM or 0.1 μM), RA (1 μM or 0.3 μM) or a combination of both. Cell growth (percent of control) was assayed in triplicate wells. The results represent the average of at least 3 independent experiments.

B. Annexin V staining of THP-1 or OCI-AML3 cells treated for 48.

C. TUNEL assay of THP-1 or OCI-AML3 cells treated for 48 hours as described. The results are the average of 3 independent experiments.

D. Flow cytometry analysis using CD11b differentiation marker on OCI-AML3 treated for 48 hours.

E. Western blot analysis for p53, P-p53, p21, or actin in THP-1 and OCI-AML3 cells treated for 48 hours.

1.2.6. Current research on other targeted therapy in AML

Novel targets are being investigated. These targets include:

Fms-Like Tyrosine Kinase 3 (FLT3) Inhibitors

Novel selective FLT3 kinase inhibitors, including G-749 and ASP2215 have lately been demonstrated to inhibit the phosphorylation of FLT3 and to decrease the resistance to drugs in pre-clinical trials. However, further clinical studies in term of efficacy are still needed (Lee, Kim et al. 2014, Altman, Perl et al. 2015).

Isocitrate DeHydrogenase (IDH) Inhibitors

New IDH1 and the IDH2 inhibitors (AG-120 and AG-221 respectively) have shown encouraging response rates in AML patients in early clinical trials (Stein, Altman et al. 2014, Platt, Fathi et al. 2015).

Immune Therapies

Innovatory therapies in AML using novel antibody-based therapies are currently under development. We can mention the CD33 as monoclonal antibody being investigated among other monoclonal antibodies such as Gemtuzumab ozogamicin (GO) that induce G₂ arrest followed by apoptosis (associated with caspase 3 activation) (Pagano, Fianchi et al. 2007, Castaigne, Pautas et al. 2012) and AMG 330 as bispecific antibodies (anti-CD33 and CD3) that led to lysis of AML cells as well as efficient activation of memory T-lymphocytes (Kufer, Kischel et al. 2013, Gasiorowski, Clark et al. 2014).

Mouse models of human AML

Animal models have been very essential for the comprehension and treatment of cancers in general and leukemias in particular. In AML, many models have been established: Transgenic knock-in, knock-out and xenograft models. NPM-1 transgenic model expressing the mutated NPM-1 under the control of myeloid-specific human MRP8 promoter (*hMRP8-NPM-1c*) showed no spontaneous AML (Cheng, Sportoletti et al. 2010). While knockout

models are not direct AML models and knock-in model (especially of *NPM-1* mutation alone) was insufficient to cause AML formation (Ishikawa, Yasukawa et al. 2005, McCormack, Bruserud et al. 2005), Xenograft models (especially the NOD/Shi-scid IL2r-gamma^{-/-} mice (NSG)) have improved the effectiveness of the engraftment of human tissues, stem cells, cord blood, bone marrow (Ito, Hiramatsu et al. 2002, Ishikawa, Yasukawa et al. 2005) as well as the unique advantage of in assessing activity of promising drugs (Cook and Pardee 2013).

1.3. Imidazoquinoxalines

For many years, Max Mousseron Institute of Biomolecules (IBMM) at the Faculty of Pharmacy, in Montpellier, France, has been synthesizing quinoxalinic compounds and imiquimod analogues, with potential antitumor activities.

These compounds are grouped in three series of chemicals:

- Imidazo[1,2-*a*]quinoxalines
- Imidazo[1,5-*a*]quinoxalines
- Pyrazolol[1,5-*a*]quinoxalines

The first group, the imidazo[1,2-*a*]quinoxalines, has been the focus of different studies that will be briefly described below.

1.3.1. Imiquimod

1.3.1.1. Clinical use and research

Imiquimod (also known as S-26308 and R-837) was FDA approved in 1997 for the treatment of external genital warts. It is a low molecular weight immune response modifier (Hemmi, Kaisho et al. 2002, Rudy 2002).

Imiquimod has been systemically administered to mice and was shown to be effective against colon, mammary and bladder carcinomas, lung sarcoma, melanoma (Sidky, Borden et

al. 1992), basal cell carcinoma (Bilu and Sauder 2003), actinic keratosis (Megyeri, Au et al. 1995), and even cutaneous B-cell lymphoma (Spaner, Miller et al. 2005). Interestingly, Imiquimod has been topically used to treat basal cell carcinoma BCC (Steinmann, Funk et al. 2000). *In vivo* studies showed that Imiquimod is a potent inducer of apoptosis in BCCs (Schön, Bong et al. 2003).

1.3.1.2. Mechanism of action

Imiquimod was shown to have antiviral and antitumor properties (Hemmi, Kaisho et al. 2002) and its mechanism of action has been widely studied.

Indirect immunomodulatory effect

The immunomodulatory component of imiquimod mode of action is characterized by the activation of the innate and the adaptive immune systems. Imiquimod binds to Toll-Like Receptors 7 and 8 (TLR-7 and 8), involved in pathogen recognition (Hemmi, Kaisho et al. 2002), thus activating the Nuclear Factor kappa B (NF- κ B) signaling pathway, leading to the release of pro-inflammatory cytokines such as interferon- α , tumor necrosis factor alpha (TNF- α) and pro-inflammatory interleukins (Reiter, Testerman et al. 1994, Stockfleth, Trefzer et al. 2003).

Direct apoptotic effect

Imiquimod has been shown to induce caspase-dependent apoptosis against melanoma *in vitro* and *in vivo*, via the mitochondrial-dependent apoptotic pathway whereby the increase in the Bax/Bcl-2 ratio induces the cytochrome c release in the cytosol, with a subsequent activation of procaspases 3 and 9. Importantly, the apoptotic mechanism is not due to membrane-bound receptors of apoptotic mediators (Schon, Bong et al. 2003). Based on the above-mentioned properties and mode of action of imiquimod, our group started synthesizing

new imidazoquinoxalines, of which imiquimod was the chemical heterocyclic model analogue.

1.3.2. Imidazo[1,2-*a*]quinoxaline

Imidazo[1,2-*a*]quinoxalines are imiquimod analogues that were shown to decrease TNF- α levels and are thought to be anti-inflammatory (Morjaria, Deleuze-Masquefa et al. 2006). These compounds were also shown to inhibit the cyclic nucleotide phosphodiesterase enzyme 4, leading to cyclic Adenosine MonoPhosphate (cAMP) accumulation inside the cell and activation of the cAMP Response Element-Binding protein (CREB) transcription factor (Deleuze-Masquefa, Gerebtzoff et al. 2004). Moreover, imidazo[1,2-*a*]quinoxalines were shown to activate the p38 MAPK and inhibit the PI3K pathways (Morjaria, Deleuze-Masquefa et al. 2006). The two compounds with most promising antitumor effects on an important number of cancers, of which melanoma, were EAPB0203 and EAPB0503, as shown by studies, compared to imiquimod or to fotemustine and approved for the treatment of metastasizing melanoma (Moarbess, Deleuze-Masquefa et al. 2008, Deleuze-Masquefa, Moarbess et al. 2009).

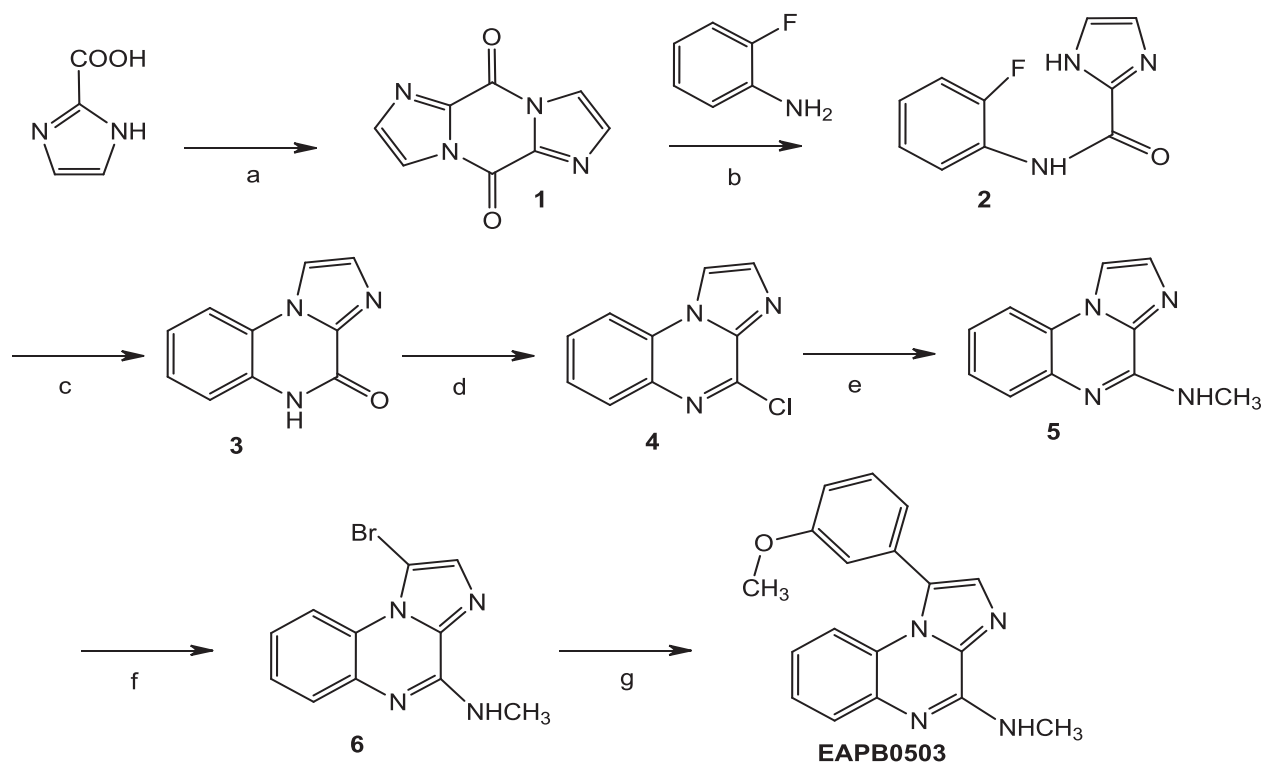
1.3.2.1. Summary of major synthesis steps

This study involved two imidazoquinoxalines, EAPB0203 and EAPB0503. EAPB0203 was synthesized as described previously by our group in Montpellier (Deleuze-Masquefa, Gerebtzoff et al. 2004). The synthesis was based on the condensation of two imidazole derivatives that were coupled to *ortho*-fluoroaniline, followed by an intramolecular cyclisation and the substitution with the appropriate amines (Moarbess, Deleuze-Masquefa et al. 2008).

The synthesis of EAPB0503 was modified and optimized to yield more purity with less synthesis time (Figure 10). Briefly, carbonyl-imidazole dimer 1 (95%) that resulted from the

bimolecular condensation of 2-imidazole carboxylic acid was coupled with *ortho*-fluoroaniline to give the intermediate 2 (90%), which was then cyclized to give compound 3 (80%). Compound 3 was treated with phosphorus oxychloride and *N,N*-diethylaniline in a sealed tube at 130°C for 15 minutes using a Biotage initiator microwave synthesizer, leading to compound 4 (90%) after column chromatography on silica gel purification. Compound 4 was coupled with methylamine in the microwave (150°C, 20 minutes) to give compound 5 (95%). The bromination of compound 5 by *N*-bromosuccinimide in the microwave (100°C, 6 minutes) led to compound 6 (85%). EAPB0503 was obtained by Suzuki coupling of compound 6 with 3-methoxyphenylboronic acid in the presence of palladium catalyst, basic conditions and under microwave assistance (140°C, 20 minutes) and was purified by column chromatography on silica gel, leading to the pure product (90%) (Figure 10). The steps a, b and c were performed in the same conditions than in the previous synthesis (Deleuze-Masquefa, Gerebtzoff et al. 2004) and the modifications affected the last four steps, greatly decreasing the total time of the synthesis. Time reduction between usual reflux and microwave conditions came as follows: step d, 6 hours to 15 minutes; step e, 20 hours to 20 minutes; step f, 2 hours to 10 minutes. Microwave-assisted chemistry was used to optimize the synthesis of EAPB0503 described by Deleuze-Masquefa *et al.*, resulting in high yielding and clean steps. Most of the intermediate compounds were obtained with yields over 80% and were pure enough to be used for the next step of the synthesis without purification.

Figure 10. Major synthesis steps of EAPB0503

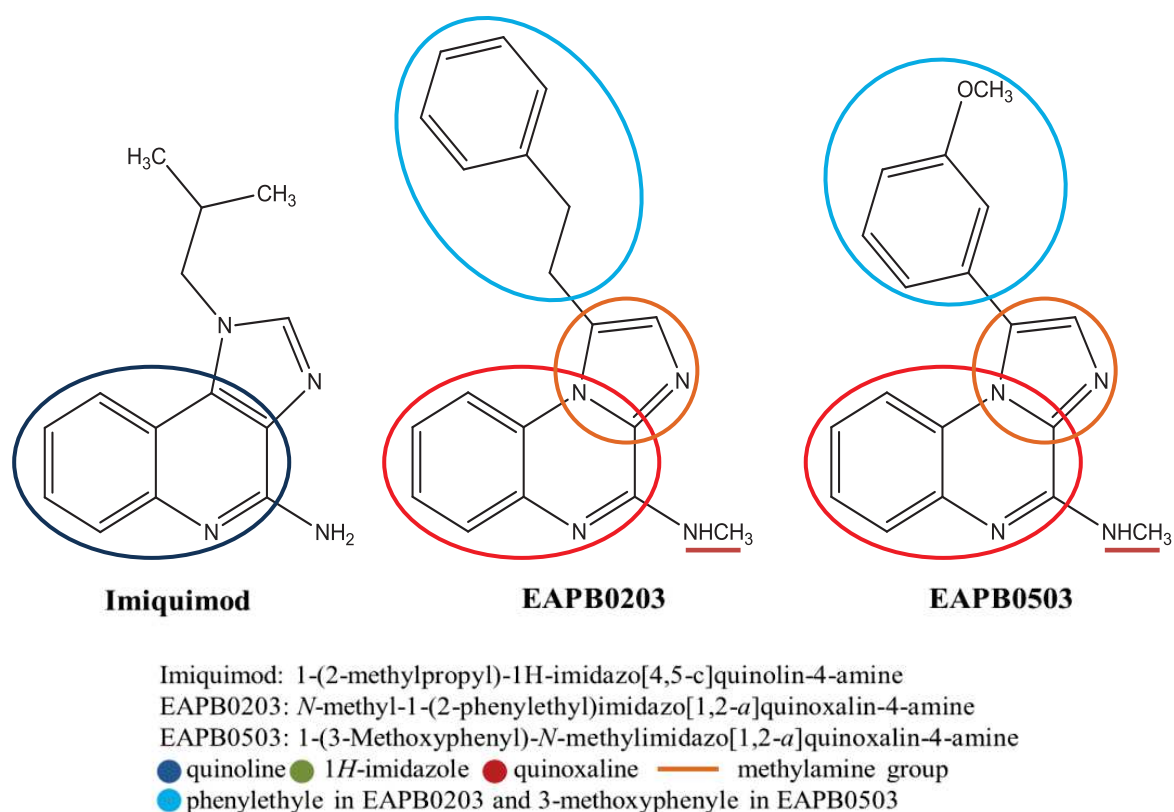


1.3.2.2. Chemical structure

Imiquimod (1-(2-methylpropyl)-1*H*-imidazo[4,5-*c*]quinolin-4-amine) is made up of a quinoline component, a 1*H*-imidazole ring and a methylpropyle group. In EAPB0203 (*N*-methyl-1-(2-phenylethyl)imidazo[1,2-*a*]quinoxalin-4-amine) and EAPB0503 (1-(3-methoxyphenyl)-*N*-methylimidazo[1,2-*a*]quinoxalin-4-amine), the quinoline part is modified into quinoxaline, maintaining, however, the 3 intracyclic nitrogens in the imidazoquinoxaline rings that might be considered as essential for the pharmacophore property of all three drugs (Moarboss, Deleuze-Masquefa et al. 2008). Imidazoquinoxalines differentiate from imiquimod by the presence of bridgehead nitrogen between the quinoxaline and imidazole rings which both constitute the tricyclic heterocyclic core of the compounds. The amine (-NH₂) group of the imiquimod molecule is replaced by methylamine (-NHCH₃) in both EAPB0203 and EAPB0503. The methylamine group seems to be essential for increased

potency of these compounds (Moarbess, Deleuze-Masquefa et al. 2008). The methylpropyle group of the imiquimod was substituted with phenylethyle in EAPB0203 and with 3-methoxyphenyle in EAPB0503, probably increasing polarity and binding properties of this newer compound. The initials EAPB stand for Equipe d'Accueil Pharmacochimie et Biomolécules (the laboratory where the compounds are being synthesized) and the numbers: "0" for imidazo[1,2-*a*]quinoxaline series, "2" for phenylethyle, "5" for methoxyphenyle and "03" for methylamine substituents (Figure 11).

Figure 11. Structural differences between imiquimod, EAPB0203 and EAPB0503.



The amine (-NH₂) group of the imiquimod molecule is replaced by methylamine (-NHCH₃) in both EAPB0203 and EAPB0503. The methylpropyle group of the imiquimod was substituted with phenylethyle in EAPB0203 and with 3-methoxyphenyle in EAPB0503.

1.3.2.2. Metabolism and pharmacokinetics

Imidazo[1,2-*a*]quinoxalines, and mostly EAPB0203, its metabolites and major oxygenated derivatives, have been characterized and identified by nuclear magnetic resonance and liquid chromatography – mass spectrometry (LC-MS) studies (Lafaille, Banaigs et al. 2012). However, the scope of our research does not include advanced chemical analysis of the compounds of interest. Briefly, EAPB0203 and EAPB0503 have been found to be metabolized inside the liver microsomes of many species, including mouse, human, dog and rat; the former two species through faster processes. Both EAPB0203 and EAPB0503 metabolites had an increased polarity compared to the parent compounds, and had mostly shorter half-lives, except for one of EAPB0503 metabolites. Metabolites issued from the *in vitro* microsomal systems were analyzed by LC-MS. The first step of EAPB0203 and EAPB0503 biotransformation is a demethylation step, leading to their two main metabolites, and the human microsome was shown to be the most effective demethylation system (Khier, Gattacceca et al. 2010). The same study reported also that around 98% of plasma EAPB0203 and EAPB0503 bind to human serum albumin, with very little drugs in the free form, quantitated after collection of human plasma proteins by ultracentrifugation. Both compounds were found to be eliminated from the plasma in two phases: an initial rapid decline phase, followed by a prolonged terminal phase. Their terminal half-lives were approximately two hours. Importantly, the systemic availability of EAPB0203 and EAPB0503, upon their intraperitoneal administration was 22.7% and 35%, respectively (Khier, Gattacceca et al. 2010).

1.3.2.3. Antitumor activity of EAPB0203

The antitumor properties of EAPB0203 were first described on the melanoma A375 cell line, as shown by corresponding *in vitro* IC₅₀ (Moarbess, Deleuze-Masquefa et al. 2008). The *in vitro* activity of EAPB0203 was also reported in T-cell lymphoma cell lines and HTLV-I-associated adult T-cell Leukemia/ lymphoma (ATL) (Moarbess, El-Hajj et al. 2008).

In vitro mechanism of action

The mechanism of action of the antitumor effect of EAPB0203 was characterized in ATL, where growth inhibition was reported upon treatment of HTLV1-positive and negative cells with micromolar concentrations of this compound (Moarbess, El-Hajj et al. 2008). EAPB0203-induced growth inhibition was further investigated and could be partially explained by a cell cycle arrest occurring at the G₂/M level. The antitumor activity was accompanied by the downregulation of anti-apoptotic proteins: IAP-1 and Bcl-xL, and the activation of the mitochondria-associated intrinsic cell death pathway characterized by cytosolic cytochrome c release and subsequent caspase 3 and 9 activation. Moreover, EAPB0203 was shown to stabilize p53 and p21 protein expression in HTLV1-positive cells by preventing their proteasome-dependent degradation (Moarbess, El-Hajj et al. 2008). By analogy to imiquimod, and though imidazoquinoxalines decrease TNF- α production, they possible retain an immunomodulatory effect.

Ex vivo human cells

Interestingly, EAPB0203 was rather tumor specific and spared normal PHA-stimulated and resting human T cells, at concentrations more elevated than the experimental ones (Moarbess, El-Hajj et al. 2008), while freshly isolated primary ATL cells, EAPB0203 induced growth inhibition that involve the p53 pathway, G₂/M cell-cycle arrest, and apoptosis, whereas no effect was observed on resting and activated normal T lymphocytes.

In vivo application

In order to translate promising *in vitro* results towards *in vivo* studies, toxicity of EAPB0203 was assessed in Sprague-Dawley rats by daily injections of 5 mg/kg inside the tail vein over five days in 100 μ L dimethylsulfoxide (DMSO). Analysis of blood samples, body weight, food and water intake, and hematological analysis of organs demonstrated no toxicity (Khier, Deleuze-Masquefa et al. 2010). In the same study, EAPB0203 proved non-toxic in Swiss mice upon intraperitoneal injections of 30 to 300 mg/kg. The lethal dose LD₅₀ in rats was 14.7 mg/kg upon intravenous administration. Bioavailability of EAPB0203 obtained after inoculation of rats with 2.5 mg/kg of EAPB0203 into the penis vein or intraperitoneally, was 22.7%. Swiss mice inoculated with M4Be melanoma cells responded in a dose-related manner to EAPB0203 better than to fotemustine (Khier, Deleuze-Masquéfa et al. 2010).

1.3.2.4. To-date research on EAPB0503

The more recently synthesized compound, EAPB0503, showed around tenfold higher potency than EAPB0203 on melanoma cell lines, and pharmacokinetic studies reported 35% bioavailability in rats upon intraperitoneal administration, (Khier, Moarbess et al. 2009). LD₅₀ in Sprague-Dawley rats was equivalent to 8 mg/kg, computed upon daily injections of 3 mg/kg EAPB0503 in DMSO into the tail vein (Khier, Moarbess et al. 2009). A recent study

revealed that EAPB0503 showed potent inhibitions of the tubulin polymerization which is correlated to the antiproliferative activities (Zghaib, Guichou et al. 2016). Therefore, the novel imidazoquinoxaline EAPB0503 is more potent than EAPB0203 *in vitro* and of higher bioavailability *in vivo*. Together, these drugs hold promising antitumor activities that should be further investigated in order to broaden the range of cancers they tackle and translated into clinical studies.

1.3.3. Imidazoquinoxalines and cancer treatment

1.3.3.1. Melanoma

Among various imidazoquinoxaline derivatives, that have been synthesized and tested to date, the two analogs of imiquimod, EAPB0203 and EAPB0503, have arisen as promising due to their *in vivo* and *in vitro* anti-tumoral activities against melanoma and T-lymphomas (Moarbess, Deleuze-Masquefa et al. 2008, Khier, Deleuze-Masquéfa et al. 2010). Some data have been published on the anti-inflammatory properties and the mechanism of action of EAPB0203 and EAPB0503 (Morjaria, Deleuze-Masquefa et al. 2006, Moarbess, El-Hajj et al. 2008). Yet, more specific molecular targets of these two analogs remain to be investigated.

1.3.3.2. Leukemia

As EAPB0503 was more recently synthesized and revealed more potent *in vitro* activity and bioavailability in rats (Khier, Gattacceca et al. 2010). Furthermore, it has been demonstrated that EAPB0503 induces a cell cycle arrest in the G2/M phase in EAPB0503-treated CML cells (Saliba, Deleuze-Masquéfa et al. 2014). H3 phosphorylation occurs at late prophase and is maintained during metaphase (Hans and Dimitrov 2001). EAPB0503 increased the PreG₀ population, suggesting a presumably apoptotic activity. PARP cleavage came after to further confirm the induction of apoptosis in treated CML cell lines (Saliba,

Deleuze-Masquéfa et al. 2014). It is previously shown that in T-cell lymphoma and HTLV-I-associated adult T-cell Leukemia/lymphoma cell lines, EAPB0203 similarly induced growth inhibition, cell cycle arrest and induction of apoptosis of malignant T cells (Moarbess, El-Hajj et al. 2008). EAPB0503 reduces the survival of CML cell by targeting the cell cycle and blocking it at mitosis. Furthermore, the degradation of oncoprotein BCR-ABL that promotes pathogenicity potentials in CML (Saliba, Deleuze-Masquéfa et al. 2014).

1.4. Apoptosis and molecular markers

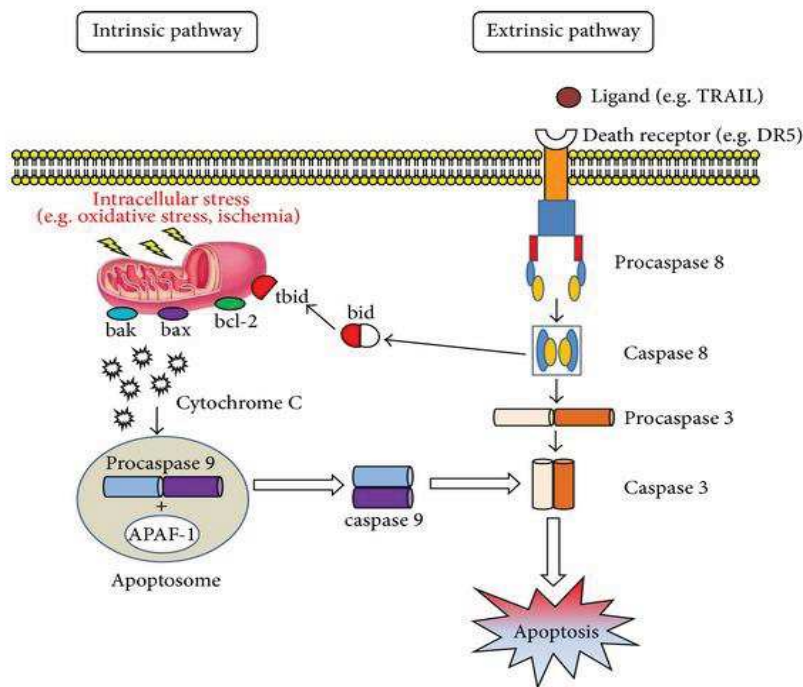
Since the mid-1980s, apoptosis has been always a highly conserved biological program that attracted remarkable research interest. Its appeal rests on intrinsic interest and the dual potential for uniting theories of pathology and inspiring new therapies in virtually every organ system (Smyth, Berman et al. 2002).

Many advances in methods development for apoptosis detection have facilitated the understanding of the signaling pathways involved in apoptosis. These markers include an increase in caspase 3 activity, poly(ADPribose) polymerase (PARP) cleavage, chromatin condensation, DNA fragmentation, decreased cellular metabolism, compromised membrane permeability, and cleavage of nuclear envelope proteins (lamins) (Smyth, Berman et al. 2002). In addition, the release of cytochrome c from mitochondria and the change in mitochondrial membrane potential are thought to occur relatively early in apoptosis (Smyth, Berman et al. 2002).

Another category of apoptosis detection focuses on signaling proteins such as the p53 tumor suppressor gene product. The accumulation of wild-type p53 following DNA damage leads to either DNA repair following cell cycle arrest or apoptosis (Smyth, Berman et al. 2002). Phosphorylation of the p53 can also be considered as the primary marker for induction of apoptosis (Thompson, Tovar et al. 2004)

Apoptosis can be induced through two alternative pathways. The first pathway (Intrinsic) is a mitochondria-dependent pathway in which the cell kills itself due to an intracellular stress. This pathway is partly affected by the Bcl family members (Bax and Bcl-2). This process promotes cytochrome c mitochondrial release in order to bind to the apoptotic protease activating factor (APAF-1) then activates procaspase 9 and caspase 3. The second pathway (extrinsic) is a death receptor-mediated pathway in which the cell kills itself due to external signals. In this pathway, procaspase 3 then caspase 3 are activated which are proteases that degrade proteins and induce apoptosis (Loreto, La Rocca et al. 2014) (Figure 12).

Figure 12. Intrinsic and extrinsic apoptotic pathways (Loreto, La Rocca et al. 2014).



1.5. Aim of the study

The potential antitumor effects of the two imidazoquinoxaline compounds, EAPB0203 and EAPB0503 were previously demonstrated to be potent on ATL (Mouarbes et al.) and on CML (Saliba, Deleuze-Masquéfa et al. 2014). In this study, we aimed to investigate the antitumor effects of these two compounds on AML.

We first started by investigating the antiproliferative properties of both compounds on four human AML cell lines (OCI-AML2, OCI-AML3, IMS-M2, KG-1 α , THP-1 and MOLM-13). We then investigated the effects of both compounds on the cell cycle progression, induction of apoptosis and on the protein levels of the constitutively active oncoprotein NPM-1 as well as other apoptotic markers.

2. Materials and Methods

2.1. Cells and drugs

2.1.1. Cell lines

KG-1 α , ML-2, THP-1 cell lines (from F. Mazurier) and IMS-M2 (from H. de Thé) were grown in RPMI-1640. OCI-AML3 cells (from D. Bouscary) were grown in MEM- α . Cells were seeded at 2×10^5 /ml. EAPB0203 or 0503 were used at 0.1 to 5 μ M, zVAD caspase inhibitor (Bachem Bioscience) at 50 μ M, and PS-341 proteasome inhibitor at 10nM.(El Hajj, Dassouki et al. 2015) Cell growth was assessed by trypan blue or CellTiter 96[®] proliferation kit (Promega).

Primary AML cells from patients' BM were extracted as described by El Hajj et al.(El Hajj, Dassouki et al. 2015) after approval by the Institutional Review Board at the American University of Beirut and after consented agreement of patients according to Helsinki's Declaration.

Table 11. AML cell lines information.

AML CELL LINE	Description	Origin & source	Morphology	p21 status	p53 status	FLT-3 status	NPM-1 status
OCI-AML3	Acute myeloid leukemia	Peripheral blood Male, 57 yo	Single, round to oval cells	Wt	wt	wt	Mutant
OCI-AML2	Acute myeloid leukemia	Peripheral blood Male, 65 yo	Single, round to oval cells	Wt	wt	wt	wt
IMS-M2	Acute myeloid leukemia	Bone marrow, Female, 59 yo	Round cells	Wt	wt	wt	Mutant
THP-1	Acute monocytic leukemia	Peripheral blood Male, 1 yo	Large, round, single-cell morphology	Wt	Mutant	wt	wt
KG-1α	Acute Erythroid leukemia	Bone marrow, Male, 59 yo	Myeloblastic	Wt	Mutant	wt	wt
MOLM-13	Acute monoblastic/monocytic leukemia	Peripheral blood, Male, 20 yo	Large, round, single-cell morphology	Wt	wt	Mutant	wt

2.1.2. Drugs

EAPB0203 and EAPB0503 were synthesized as described by Deleuze-Masquefa *et al.* (Deleuze-Masquéfa, Gerebtzoff et al. 2004, Deleuze-Masquefa, Moarbess et al. 2009). The synthesis of EAPB0503 has recently been optimized by microwave-assisted chemistry (Zghaib, Guichou et al. 2016). EAPB0203 and EAPB0503 powders were prepared in dimethylsulfoxide (DMSO) (Amresco, OH, USA) as stock solutions at 0.1 M and aliquots were stored at -20°C. Working solutions of the compounds were freshly prepared in RPMI

1640 (Lonza, Belgium) for treatment of THP-1, MOLM-13, and KG1- α or in MEM- α (Gibco by life technologies, UK).

2.1.3. Generation of wt- or NPM-1c expressing cells

GFP wt- or *NPM1c* inserts were amplified and ligated into a pBye lentiviral vector by EcoRI site. Stable OCI-AML2 expressing wt- or NPM1c were generated by lentiviral transduction followed by blasticidin selection. GFP positive cells were sorted using a FACS Aria SORP (Becton Dickinson, BD) and grown in MEM- α , before cell growth assessment.

HeLa cells were transfected with pcDNA-HA expressing wt- or *NPM-1c* (from G. Tell)(Vascotto, Lirussi et al. 2014) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations and grown in DMEM.

2.1.4. Xenograft animal studies

NOD/Shi-*scid* *IL2r-gamma*^{-/-} mice (NSG) were obtained from Jackson Laboratories (USA). Mice protocols were approved by the Institutional Animal Care and Utilization Committee of the American University of Beirut. 1×10^6 OCI-AML3 or THP-1 cells were injected into the tail vein of 8-week-old females (5 mice per group). On day 5 post AML injection, mice were treated with 15 mg/kg of EAPB0503 for 5 days a week, over a period of 2 weeks. EAPB0503 was dissolved in DMSO and diluted in equal volume of lipofundin (vehicle) before intraperitoneal administration to mice.(Moarbess, Deleuze-Masquefa et al. 2008, Khier, Gattacceca et al. 2010)

2.1.5. Cell culture

KG-1 α , ML-2, THP-1 and IMS-M2 cell lines were grown in complete RPMI-1640. OCI-AML3 and OCI-AML2 cells were grown in complete MEM- α supplemented with glutamax and 20% Fetal Bovine Serum. Cells were seeded at 2×10^5 /ml. EAPB0203 or 0503 were used at 0.1 to 10 μ M, zVAD caspase inhibitor (Bachem Bioscience) at 50 μ M, and PS-

341 proteasome inhibitor at 10 nM. Primary AML cells from bone marrow of patients were extracted as described by (El Hajj, Dassouki et al. 2015) after approval by the Institutional Review Board at the American University of Beirut and after consented agreement of patients according to Helsinki's Declaration. Cell concentration of 200,000 cells/ml were used for all experiments.

2.2. Cell viability

2.2.1. Proliferation assay

Cells were cultured in were grown in RPMI-1640 and MEM- α in the presence and absence of both compounds, EAPB0203 and EAPB0503. . We used drug concentrations ranging from 0.1 to 5 μ M. Cytotoxicity was analyzed at three time points (24, 48 and 72 hours) using the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). Cell growth was assessed by trypan blue or using the CellTiter 96 proliferation kit (Promega).

2.3. Flow cytometry

2.3.1. Cell cycle analysis

Cell cycle analysis was performed using propidium iodide (PI) staining and a BD FACS instrument (Becton Dickinson, New Jersey, USA). Briefly, control and treated cells were collected 48 hours post-treatment, washed with Phosphate-Buffered Saline (PBS), fixed in 80% ethanol and stored at -20°C . Subsequently, pellets were collected, rinsed with PBS and treated with 100 μ l of DNase-free ribonuclease A (Roche Applied Science, Germany) at a final concentration of 200 μ g/ml, and then stained with 50 μ g/ml PI (Sigma-Aldrich Co, Missouri , USA). Cell cycle analyses were performed by flow cytometry.

2.3.2. Annexin V staining

Both phosphatidyl-serine (PS) exposure and viability (permeability) were assessed using annexin V-fluorescein isothiocyanate (FITC) kit (BD Pharmingen, Santiago, California, USA) according to manufacturer's instructions. Briefly, Annexin V binds to PS, which, in apoptotic cells, flips to the outer leaflet of the plasma membrane. PI will only enter dead cells. Cells were exposed for 24 hours to 1 μ M of EAPB0503, and the Annexin V/PI labeling of cells was performed and analyzed by flow cytometry.

2.3.3. Mitochondrial membrane potential (MMP)

Mitochondrial membrane potential was assessed by the ability of cells to retain rhodamine (R123) (Sigma-Aldrich Co, St Louis, Missouri, USA). R123 is a cationic fluorescent dye that accumulates in active mitochondria with high membrane potentials. EAPB0503-treated cells were washed twice in 130 mM NaCl, 5 mM KCl, 1 mM Na₂HPO₄, 1 mM CaCl₂, 1 mM MgCl₂, and 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer, pH 7.4, and were then stained with 5 μ M R123 (Sigma-Aldrich, St Louis, MO, USA) for 30 minutes at 37°C. Cells were washed twice with the former buffer. R123 was excited at 488 nm, and fluorescence emission at 525 nm was assessed using flow cytometry.

In all flow cytometry experiments, a BD FACS instrument (Becton Dickinson, New Jersey, USA) was used. For each sample, 10000 events were acquired and results were analyzed using the FlowJo software (FlowJo LLC, Oregon, USA).

2.3.4. Human CD45 staining

BM from the femurs and tibias of euthanized animals were flushed at the end of week 3 post-AML inoculation. Cell surface staining was performed on 100 μ L of sample using 20 μ L of the anti-human CD45 PerC-P antibody (BD#345809). After incubation for 15 min in dark, erythrocytes were lysed using 1 mL FACS Lyse (BD). Labeled samples were washed twice and analyzed on a Guava flow cytometer.

2.4. Immunoblot analysis

Cells were treated with EAPB0203 or 0503 for 48 hours. Total protein lysates were obtained by adding 100 μ l of Laemmli sample buffer (BioRad laboratories, Hercules, California, USA) to the harvested pellets. Samples were boiled and quantified using a Nanodrop (Thermo Scientific, ND-1000, Massachusetts, USA). An equal amount of total cell lysate (50 μ g) was then loaded on 12% SDS-PAGE gel and transferred onto nitrocellulose membranes, which were blocked and probed against the following antibodies: Poly ADP-ribose polymerase (PARP) (sc-7150, Santa Cruz Biotechnology, Santa Cruz, California, USA), p53 (sc-126, Santa Cruz Biotechnology, Santa Cruz, California, USA), phosphorylated p53 (#9284, New England Biolabs, Beverly, Massachusetts, USA), p21 (sc-1397, Santa Cruz Biotechnology, Santa Cruz, California, USA), *NPM-1* (ab52644, Abcam, Cambridge, Massachusetts, USA), followed by incubation with the appropriate HRP-conjugated secondary antibodies. Loading control was achieved by probing the membrane with the mouse monoclonal horseradish peroxidase (HRP)-conjugated Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (Abnova Corporation, Taipei, Taiwan) or beta-actin (ab8227, Abcam, Cambridge, Massachusetts, USA). Immunoblots were detected by chemoluminescence using the luminol detection kit (Santa Cruz Biotechnology, Santa Cruz,

California, USA) and images were captured using the XOMAT or BioRad Chemidoc MP system (170-8280, Bio-Rad Laboratories, Inc., Hercules, California, USA).

2.5. Immunofluorescence microscopy

OCI-AML3, THP-1 cells or blasts from patients were spun down onto glass slides, then fixed and permeabilized with ice cold methanol for 30 minutes. Immunostaining was performed using a monoclonal antibody against anti-B23 *NPM-1* recognizing both *wt* and *NPM-1c* (sc-47725, Santa Cruz Biotechnology, Santa Cruz, California, USA) and a polyclonal antibody against the nucleolar marker fibrillarin (ab5821, Abcam, UK). Primary antibodies were revealed by Alexa-Fluor 488- or 594-labeled secondary antibodies (sc-2030 and sc-2031, Santa Cruz Biotechnology, Santa Cruz, California, USA). Images were acquired using the Zeiss Laser Scanning Microscope (LSM 710, Zeiss, Oberkochen, Germany), operated by Zen 2009 software (Carl Zeiss).

2.6. *NPM-1* transcript expression

2.6.1. Real time PCR analysis

Total RNA was extracted from control and EAPB0503-treated cells, using the TRIzol extraction method. RNA concentration was quantified using the Nanodrop spectrophotometer (Thermo Scientific, ND-1000, MA, USA). cDNA was then prepared using the cDNA synthesis kit (Thermo Scientific, MA, USA) as per the manufacturer's instructions. Syber green quantitative real-time (RT)-PCR was performed using the BIORAD machine (CFX96 Optics Module, Serial No. 785BR04788). Primers for Nucleophosmin-1 are directed against *NPM-1wt* (Forward 5'- ACTCCACCCTTTGCTTGGTTT, Reverse 5'- TTTGTCTCCCCACCATTTCC) or mutant *NPM-1* (Forward 5'- AAAGGTGGTTCTCTTCCCAAAGT, Reverse 5' CTCCTCCACTGCCAGACAGA). Primers for the housekeeping gene are directed against Glyceraldehyde-3-Phosphate

dehydrogenase GAPDH (Forward 5'-CATggCCTTCCgTgTTCCTA-3', Reverse 5'-CCTgCTTCACCACCTTCTTgAT-3'). Individual reactions were prepared with 0.25 μ M of each primer, 150 ng of cDNA and SYBR Green PCR Master Mix to a final volume of 10 μ l. PCR reaction consisted of a DNA denaturation step at 95°C for 3min, followed by 40 cycles (denaturation at 95°C for 15 sec, annealing at 56°C for 60 sec, extension at 72°C for 30 sec). For each experiment, reactions were performed in duplicates and expression of individual genes was normalized to GAPDH Ct values. The threshold cycle (Ct) corresponds to the cycle at which there is a significant detectable increase in fluorescence. So, comparing the Ct values of a target gene (*NPM-1wt* or mutant *NPM-1*) to that of an endogenous reference gene (GAPDH), allows the gene expression level of the target gene to be normalized to the amount of input RNA or cDNA. This is mainly done by calculating Δ Ct ($Ct_{\text{target gene}} - Ct_{\text{GAPDH}}$). Thereafter, $\Delta\Delta$ Ct is calculated according to the Livak method: $2^{-\Delta\Delta Ct}$ to obtain the percentage of expression (Schmittgen and Livak, 2008).

2.7. Statistical analysis

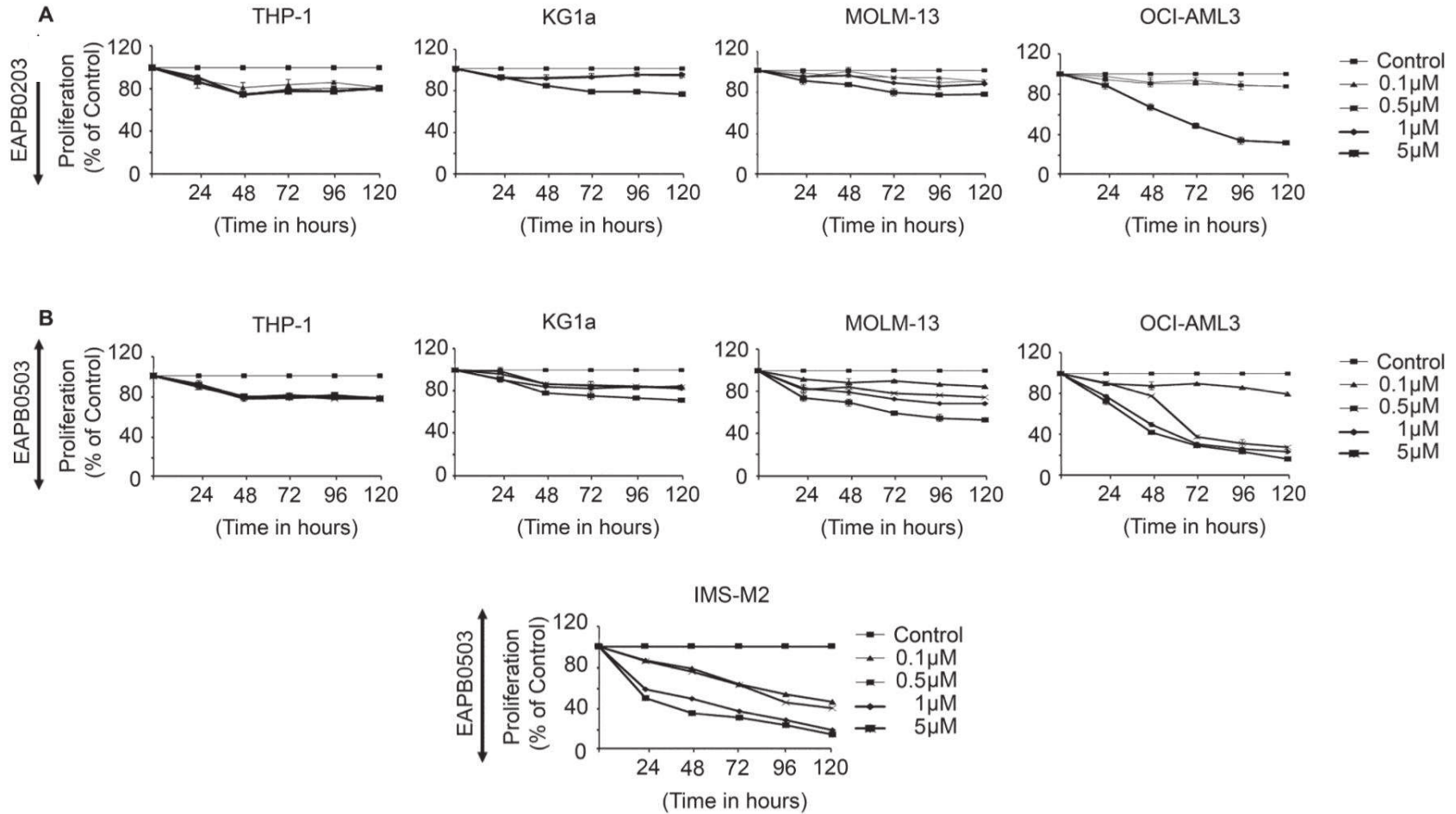
All the experiments described in this study were run in at least 3 independent experiments. Data were reported as the average \pm standard deviations. Statistical analysis was done using *Student's t* test *p*-value of less than 0.05 was considered as significant.

3. Results

EAPB0503 but not EAPB0203 induces growth arrest in *NPM-1* mutant AML cells

We used three *wt-NPM-1* cell lines (THP-1, KG1- α , and MOLM-13) and the two available *NPM-1c* AML cells (OCI-AML3 and IMS-M2) to test for EAPB0203 and EAPB0503 effects on cell growth and viability. We tested a range of 0.1 to 5 μ M drug concentrations and assessed cell growth for 5 days post-treatment (p.t.). Both treatments resulted in a pronounced time-dependent growth inhibition in OCI-AML3 cells (Figure 13A, 13B). 5 μ M of EAPB0203 resulted in significant OCI-AML3 growth inhibition ($p < 0.05$) starting 72h p.t. Strikingly, EAPB0503 was more potent and resulted in significant growth inhibition at 0.1 μ M starting 96h p.t. ($p < 0.001$). Similarly, a significant growth inhibition was obtained on both OCI-AML3 and IMS-M2 at 0.5 μ M starting 72h p.t. ($p < 0.001$), and at 1 and 5 μ M starting 24h p.t. ($p < 0.05$ and < 0.001 respectively) (Figure 13B). Importantly, an IC₅₀ of 1 μ M in OCI-AML3 or IMS-M2 cells was achieved 2 days after treatment with EAPB0503 ($p < 0.05$ and $p < 0.001$ respectively) as compared to 5 μ M after treatment with EAPB0203 in OCI-AML3 (Figure 13A, 13B). This higher potent effect of EAPB0503 when compared to EAPB0203, is in line with previously reported results (Saliba, Deleuze-Masquéfa et al. 2014). THP-1 and KG1- α cells were minimally sensitive to the compounds with only around 20% growth inhibition, even at 5 days p.t. (Figure 13A, 13B). MOLM-13 cells were also minimally sensitive to EAPB0203, but displayed around 50% growth inhibition upon 72h p.t. with EAPB0503 (Figure 13A, 13B). This percentage did not get more pronounced even 5 days p.t., whereby the only significant result was obtained at 1 or 5 μ M and starting 120h or 72h p.t. respectively ($p < 0.05$) (Figure 13B).

Figure 13. EAPB0503 induces growth inhibition in *NPM-1* mutant AML cell line.

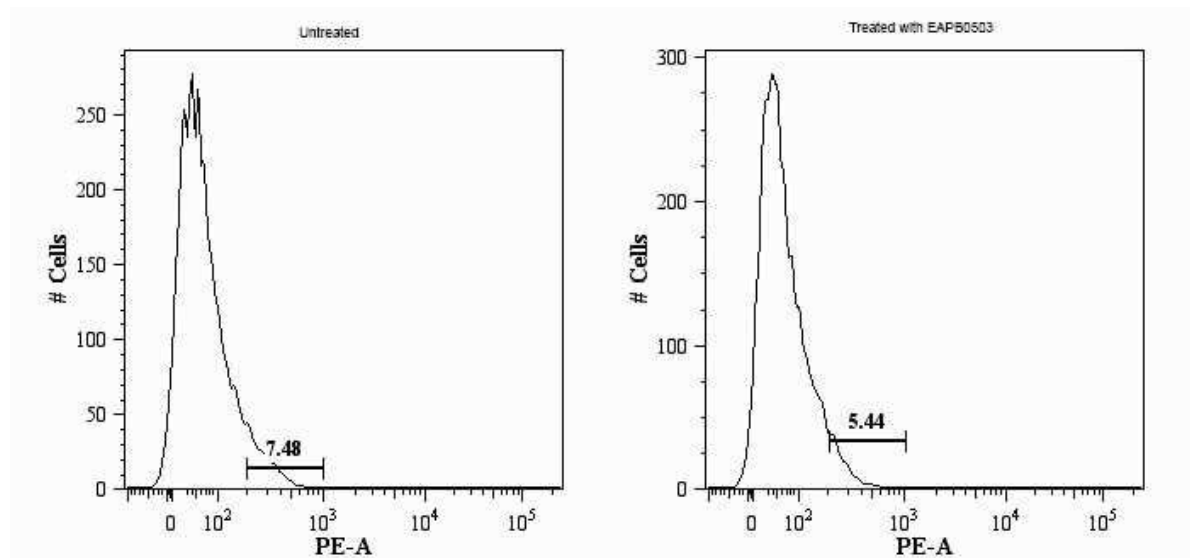


AML cell lines with normal *NPM-1* (THP-1, KG1- α and MOLM13) and *NPM-1c* (OCI-AML3 and IMS-M2) were treated with increasing concentrations (0.1 μ M to 5 μ M) of **A.** EAPB0203 and **B.** EAPB0503 for 24, 48, 72, 96 or 120 hours.

No toxicity can refer to EAPB0503 treatment on Mononuclear Cells (MNCs)

Mononuclear cells (MNCs) were extracted from mobilized peripheral blood of deceased subjects and maintained in StemSpan media (H3000, Stem Cell Technologies) supplemented with 80ng/ml of FMS-like tyrosine kinase-3 (Flt-3), 25ng/ml of Stem Cell Factor (SCF), 10ng/ml of Interleukin 3 (IL-3) and 100ng/ml of IL-6. Cells were maintained at 37 °C in a humidified incubator containing 5% carbon dioxide. Treatment with EAPB0503 was performed for 24h and staining with an anti-hCD34 antibody was performed. Flow cytometry analysis shows minimal effect of the drug on CD34+ cells. Briefly, the untreated MNCs, the percentage of CD34+ was 7.5%. Upon treatment EAPB0503, this percentage slightly decreased to reach 5.4% (Figure 14).

Figure 14. EAPB0503 induces a very slight effect on CD34+ hemopoietic stem cells

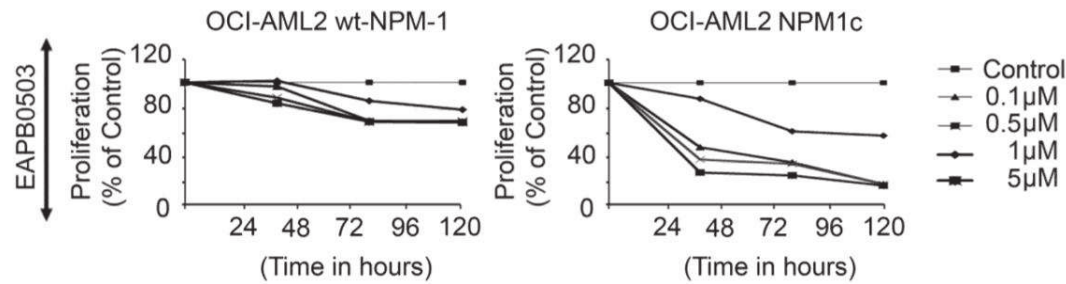


Mononuclear cells treated with a concentration of 1 μ M of EAPB0503 for 24 hours and staining with anti-hCD34 then analyzed by flow cytometry.

Introduction of *NPM-1c* in wt-NPM-1 expressing cells sensitizes them to EAPB0503

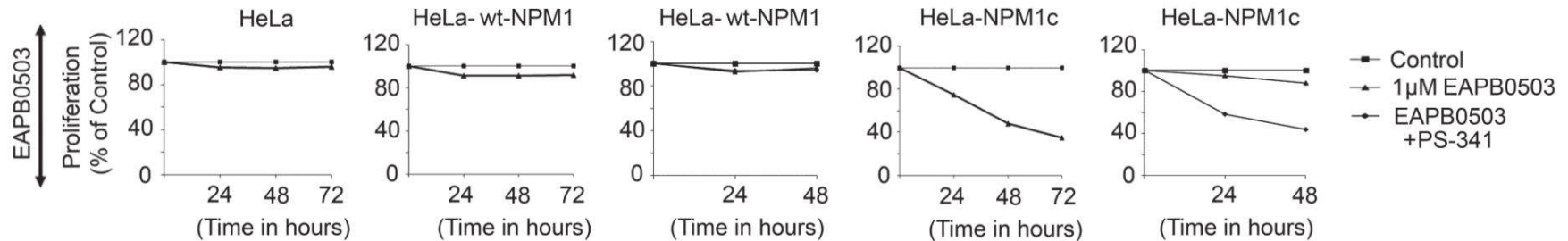
To examine whether the solely observed growth inhibition in NPM-1c cell lines is due to NPM-1 mutation, we introduced NPM-1c to wt-NPM-1 expressing cells and checked for their sensitivity to EAPB0503. We used the wt-NPM-1 expressing AML cell line, OCI-AML2, and generated by lentiviral transduction followed by blasticidin selection, cells stably expressing either GFP tagged wt-NPM-1 or NPM-1c. GFP positive cells were sorted and a range of 0.1 to 5 μ M EAPB0503 was tested to assess cell growth over 5 days p.t. Interestingly, stable expression of NPM-1c in OCI-AML2 resulted in a significant pronounced growth inhibition at 0.1 μ M starting 72h p.t. and at 0.5, 1 and 5 μ M starting 48h p.t. ($p < 0.05$) (Figure 16). A minimal effect was observed in wt-NPM-1 OCI-AML2, whereby a maximum of non-significant 30% growth inhibition was obtained 48h p.t. with 0.5, 1 and 5 μ M (Figure 15). Similar results were obtained in HeLa cells whereby 1 μ M induced growth arrest starting 48h p.t. in HA expressing NPM-1c ($p < 0.001$) but not wt-NPM-1 transfected cells (Figure 16). This growth inhibition was reversed upon adding PS-341 in NPM-1c ($p < 0.05$) but not wt-NPM-1 expressing cells at both 24 and 48h p.t. (Figure 16). Our results strongly suggest that introducing NPM-1c into cells harboring wt-NPM-1 sensitizes them to EAPB0503. Given its potency, especially in NPM-1c AML cells, only EAPB0503 was adopted at its IC₅₀ of 1 μ M, for the remainder of the study.

Figure 15. Introduction of NPM-1c in OCI-AML2 cells sensitizes them to EAPB0503



Stably transfected OCI-AML2 with GFP *wt-NPM-1* or *NPM-1c* were treated with increasing concentrations (0.1 μM to 5 μM) of EAPB0503 for 24, 48, 72, 96 or 120 hours. The results represent the average of at least three independent experiments.

Figure 16. Introduction of NPM-1c in HeLa cells sensitizes them to EAPB0503 and addition of PS-341 reverses the growth inhibition.

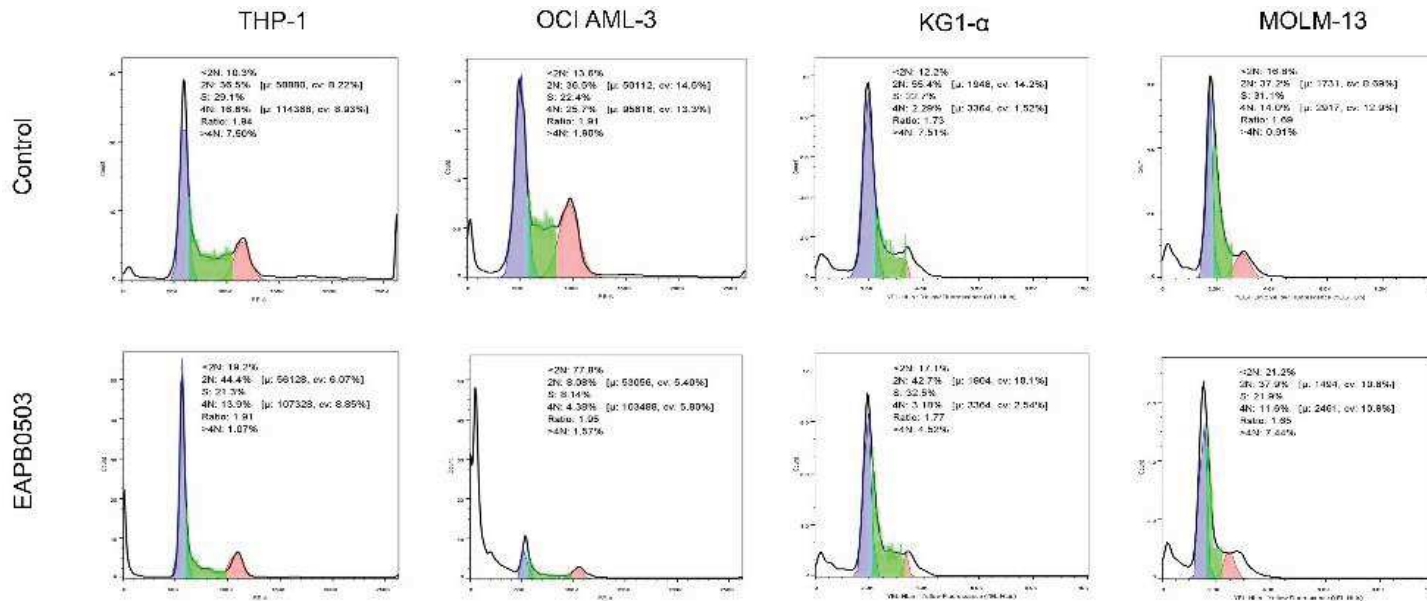


HeLa cells transfected with HA tagged *wt-NPM-1* or *NPM-1c* were treated with 1 μM of EAPB0503 alone, or in combination with 10 nM PS-341 for 24, 48 and 72h as indicated. Cell growth (% of control) was assayed in triplicates. The results represent the average of at least three independent experiments.

EAPB0503 induces massive apoptosis in *NPM-1* mutant AML cells

In order to investigate the mechanisms involved in EAPB0503-induced growth inhibition and cell death, cell cycle analysis was performed after treating wt or mutant *NPM-1* AML cells with EAPB0503 at 1 μ M (representing the IC50 of the drug for the most sensitive AML cell line) up to 48 hours (Figure 17).

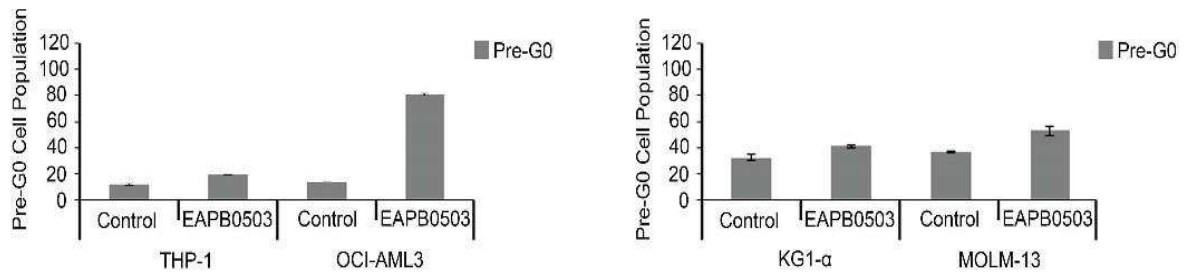
Figure 17. Cell cycle analysis of control and treated AML cells.



Cells were treated with IC50 doses of EAPB0503 and harvested after 48 hours post-treatment, stained with PI. Cell cycle analysis was assessed by flow cytometry and analyzed using Flowjo software. Graphs display the different phases of the cell cycle. G₀/G₁ phase (in blue), S phase (in green) and G₂/M phases (in red).

A sharp increase in the pre-G₀ cell percentage reaching more than 80% was obtained upon treatment of OCI-AML3 with EAPB0503. Minimal effect was observed on the three *wt-NPM-1* AML (THP-1, KG1- α or MOLM-13) (Figure 18).

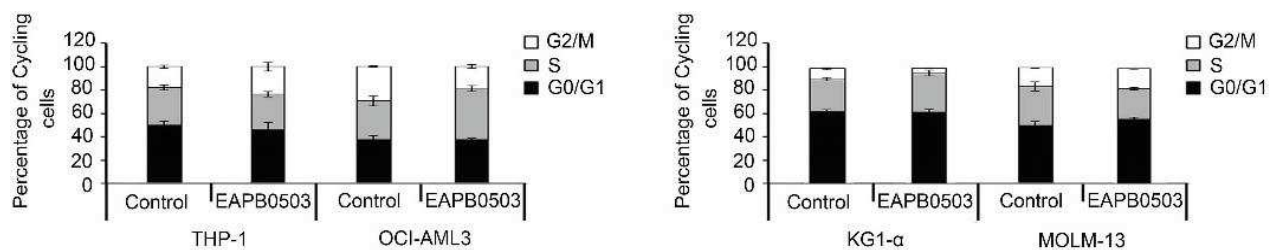
Figure 18. EAPB0503 induces massive accumulation of *NPM-1* mutant AML cells in pre-G₀.



PI-staining and cell cycle analysis show an accumulation of OCI-AML3 cells in PreG₀ upon treatment with EAPB0503 for 48 hours. Histograms represent averages \pm SD of 3 independent experiments and displays cells in preG₀.

No major variation in cell cycle distribution was observed in all tested AML cells, untreated or treated with EAPB0503 (Figure 19), showing that the drug is mostly inducing pre-G₀ accumulation in *NPM-1* mutant AML, without affecting the other cell cycle phases.

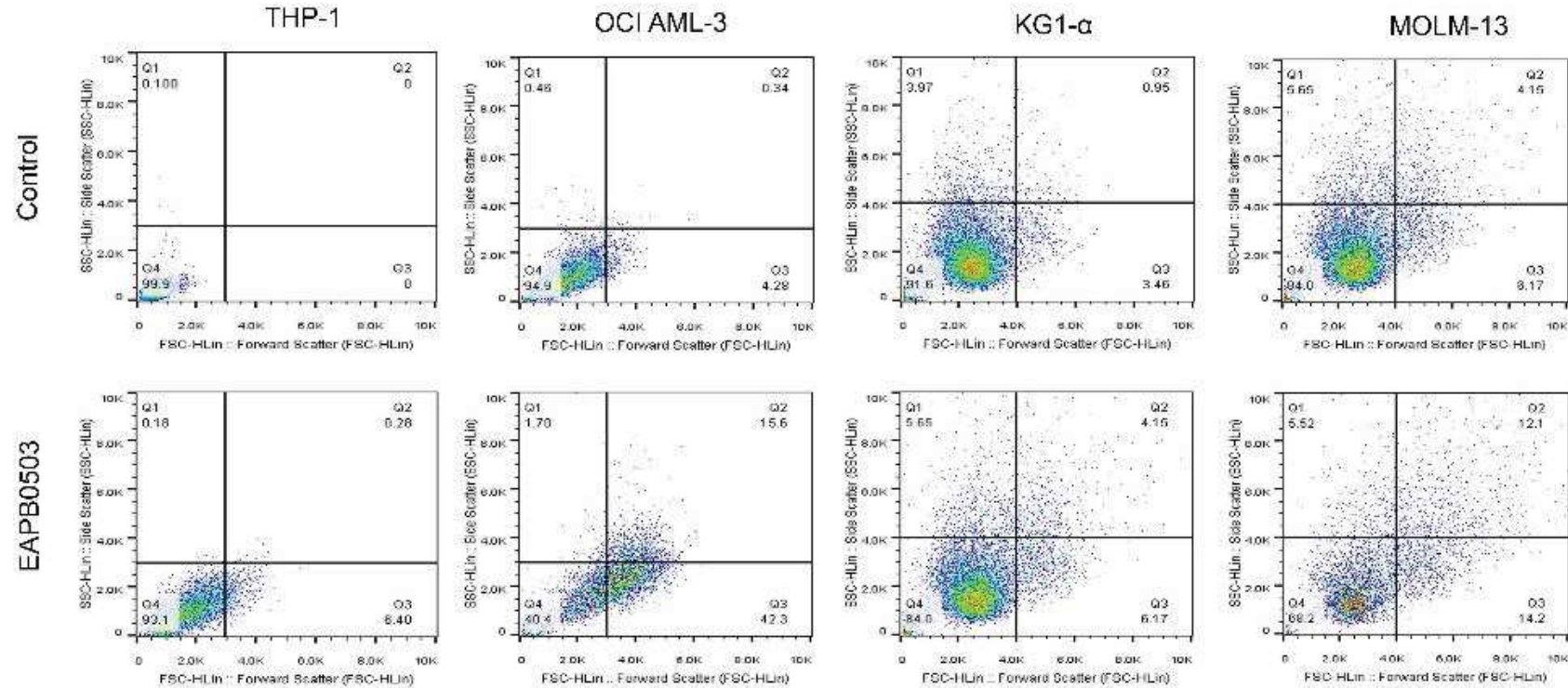
Figure 19. Cell cycle distribution of control and treated AML cells.



Percentage of cycling cell populations following PI staining upon treatment of AML cells for 48 hours as described above. Histograms represent the relative distribution of non-apoptotic cells between G₀/G₁ phases (black boxes), S phase (gray boxes) and G₂/M phases (white boxes). Results of one representative of three independent experiments are shown

To confirm apoptosis, Annexin V/PI labeling was performed on control and treated cells for 24 hours (Figure 20).

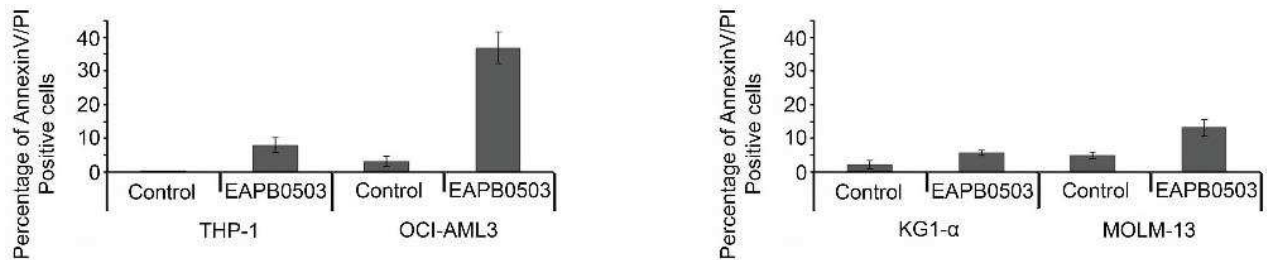
Figure 20. Annexin V/PI labeling of control and treated AML cells.



Individual flow cytometry graphs from a PI staining representative experiment of AML cell lines (THP-1, OCI AML-3, KG1-α and MOLM-13) treated with 1 μ M of EAPB0503 for 48 hours.

A significant increase of 40% in Annexin-V positivity was only observed in OCI-AML3 cells treated with 1 μ M EAPB0503 for 24h ($p < 0.005$). In contrast, the three tested *NPM-1wt* cell lines remained virtually Annexin V-negative upon treatment with the drug (Figure 21).

Figure 21. Percentages of pre-apoptosis in control and treated AML cells.

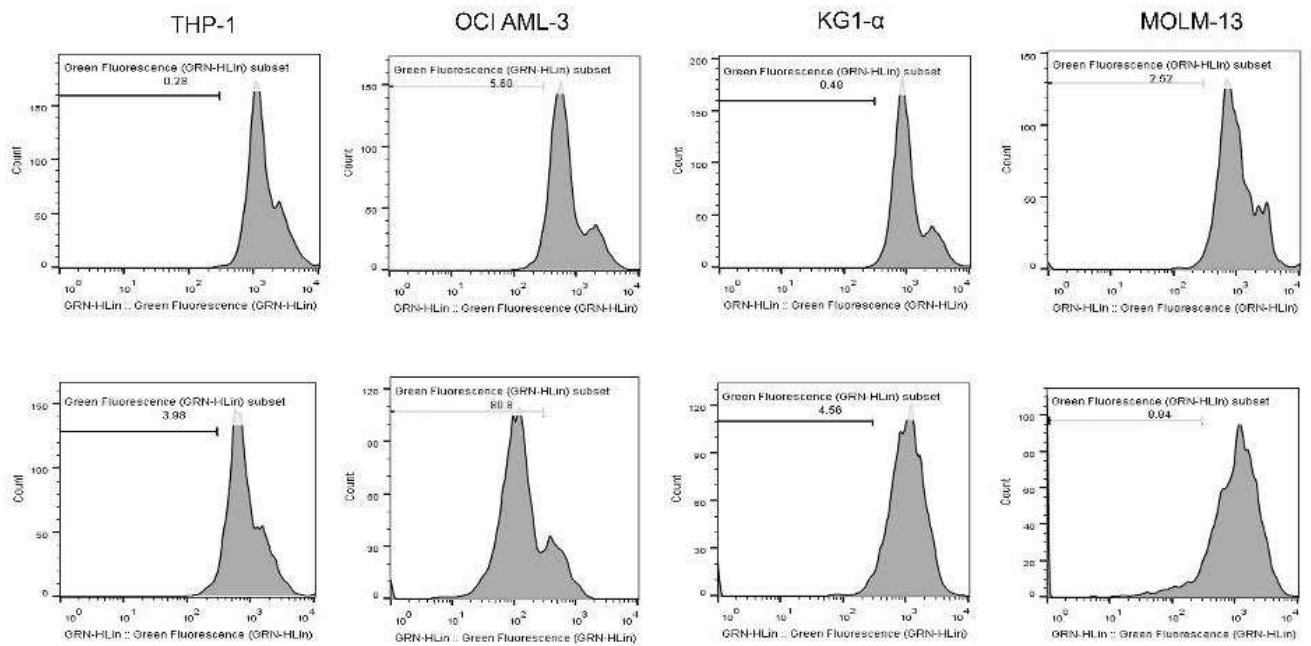


Annexin V staining of AML cells treated for 48h as described above. The results depict averages \pm SD of 3 independent experiments.

EAPB0503-induced apoptosis in *NPM-1* mutant AML cells involves dissipation of mitochondrial membrane potential (MMP) and caspase activation

Disruption of the MMP is one of the earliest and critical steps in the intrinsic apoptotic cascade (Horita, Andreu et al. 2000). To check whether EAPB0503-induced apoptosis was associated with dissipation in MPP, we measured MMP in AML cells, untreated and treated with EAPB0503 for 2 days (Figure 22).

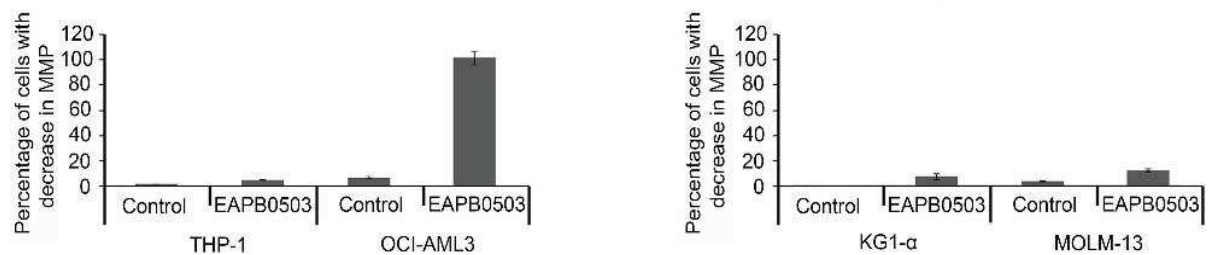
Figure 22. Dissipation of mitochondrial membrane potential (MMP) in control and treated AML cells.



Individual flow cytometry graphs from a Mitochondrial Membrane Potential staining representative experiment of AML cell lines (THP-1, OCI AML-3, KG1- α and MOLM-13) treated with 1 μ M of EAPB0503 for 48 hours.

Consistent with increased apoptosis, EAPB0503 resulted in the dissipation of MMP as shown by the inability of treated OCI-AML3 cells to actively retain the R123 dye inside their mitochondria (Figure 23).

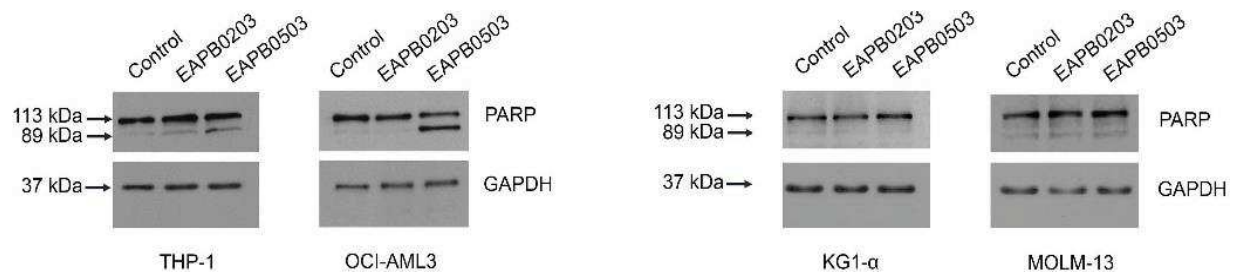
Figure 23. Percentage of MMP dissipation in control and treated AML cells.



Following treatment with AML cells as described above and Rhodamine 123 staining, R123 was excited at 488 nm, and fluorescence emission at 525 nm was assessed by flow cytometry. Histograms display averages \pm SD of 3 independent experiments.

Conversely, the three *NPM-1wt* AML cells did not show any loss of MMP up to 48 hours of treatment (Figure 23). We then assessed the effect of the dissipation of the mitochondrial membrane potential in EAPB0503-treated AML cells on the caspase cascade. In OCI-AML3, apoptosis was associated with the activation of caspases as evident by PARP cleavage (113 kDa) into its death-associated fragment (89 kDa) (Figure 24). This was detected 48 hours post-treatment in *NPM-1* mutant cells when treated with EAPB0503 but not EAPB0203, and to a much lesser extent in the three *NPM-1wt* AML cells treated with either drugs (Figure 24).

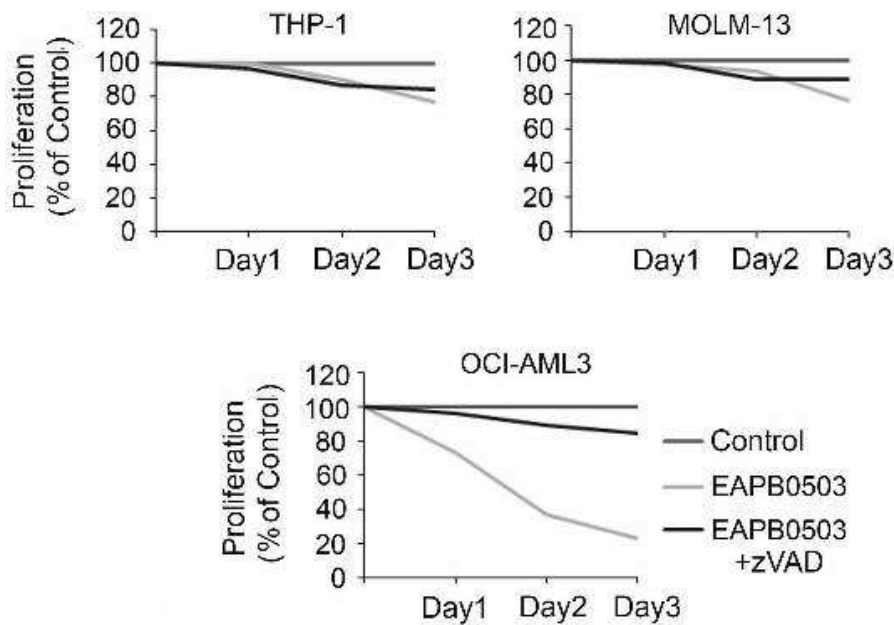
Figure 24. Western blot analysis of PARP in control and treated AML cells.



Western blot analysis for PARP upon 48-hour treatment with EAPB0203 and EAPB0503 of AML cells. Blots are representative of 3 independent experiments.

Interestingly, EAPB0503-induced growth inhibition of OCI-AML3 was reversed by the general caspase inhibitor zVAD, while in *NPM-1*wt cells (THP-1 and MOLM-13), addition of zVAD had no effect (Figure 25).

Figure 25. MTT assay on control and treated AML cells *ex-vivo*.



Proliferation assay following treatment of AML cells (THP-1, MOLM-13 and OCI-AML3) with 1 μ M of EAPB0503 alone or in combination with 50 μ M of the general caspase inhibitor zVAD for 24, 48 and 72 hours. Cell growth is represented as (% of control) as indicated.

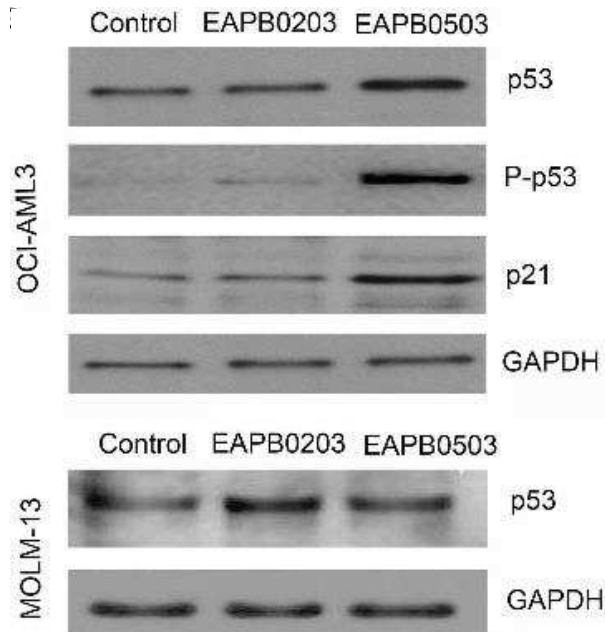
Altogether, these results demonstrate the involvement of caspase activation in EAPB0503-induced growth arrest in *NPM-1* mutant AML cells only.

EAPB0503 treatment activates p53 signaling in *NPM-1* mutant AML cells

To decipher whether the EAPB0503-mediated growth inhibition and cell death is mediated by the p53 pathway, p53 protein levels were monitored 48h hours following 1 μ M treatment of AML cell lines with EAPB0203 or EAPB0203 and compared to untreated controls. EAPB0503 induced a substantial upregulation of total p53 protein levels, and of the

p53 phosphorylated form exclusively in the mutant *NPM-1* mutant OCI-AML3 cell line while no effect was observed upon treatment of these cells with EAPB0203 (Figure 26).

Figure 26. Western blot analysis for p53, P-p53, p21 and GAPDH in AML cells.



Western blot analysis for p53, P-p53, p21 and GAPDH in OCI- AML3 and MOLM-13 cells treated for 48 hours as described.

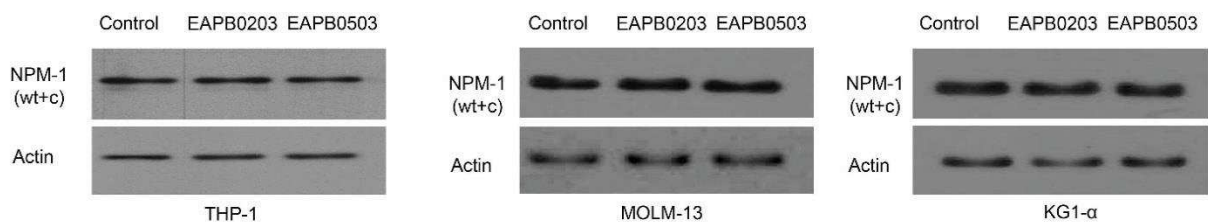
In accordance with p53 activation and upregulation, p21 protein levels were upregulated in OCI-AML3 treated with EAPB0503 but not EAPB0203 (Figure 25). Since p53 is mutated in both THP-1 and KG-1 α cell lines (Sugimoto, Toyoshima et al. 1992), we only tested p53 in the *NPM-1wt* MOLM-13 cell line and found that p53 protein levels remained unchanged upon treatment with either EAPB0203 or EAPB0503 (Figure 26).

Altogether, these results show that EAPB0503 is a potent inducer of apoptosis exclusively in *NPM-1* mutant AML cells.

EAPB0503 induces the proteasomal degradation of mutant *NPM-1* and restores the nucleolar localization of *NPM-1wt* in mutant *NPM-1* AML cells

Given the selective effect of EAPB0503 on *NPM-1* mutant AML cells, we investigated the ability of this compound to induce degradation of the mutant *NPM-1* oncoprotein. No effect of EAPB0203 or EAPB0503 on *NPM-1* expression was observed in THP-1, MOLM-13 and KG1- α cells (Figure 27).

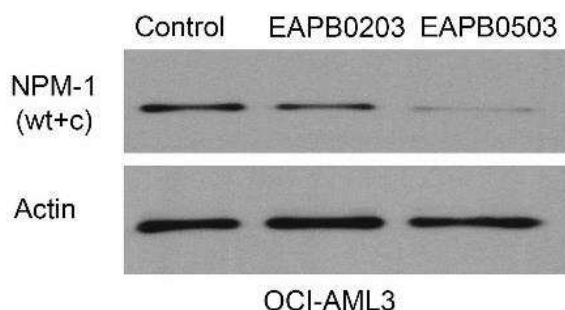
Figure 27. Western blot analysis for *NPM-1* (*wt+c*) in AML cells.



Western blot analysis for NPM-1 recognizing both wild-type and mutated NPM-1 (*wt+c*), and actin in AML cell lines with normal NPM-1 (THP-1, MOLM-13 and KG1- α).

In contrast, EAPB0503 but not EAPB0203, triggered *NPM-1* downregulation in OCI-AML3 cells, as assessed with an antibody detecting both *wt* and mutant proteins (Figure 28).

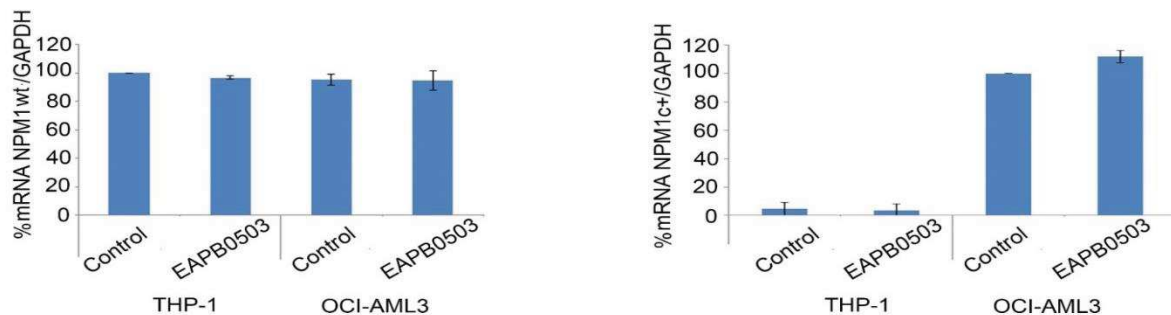
Figure 28. Western blot analysis for NPM-1 (*wt+c*) in OCI-AML3 cells.



Western blot analysis for NPM-1 recognizing both wild-type and mutated NPM-1 (*wt+c*), and actin in the *NPM-1* mutant OCI-AML3 cell line treated with 1 μ M of EAPB0203 or 0503 for 48 hours as indicated.

Collectively, this suggests that mutant *NPM-1* is the primary target of EAPB0503. Downregulated protein levels might be due to protein degradation or to decreased gene expression. Transcript levels of both *wt* and mutant *NPM-1* were not affected by treatment (Figure 29) showing that *NPM-1* downregulation is not occurring at the transcriptional level, but rather at the protein level.

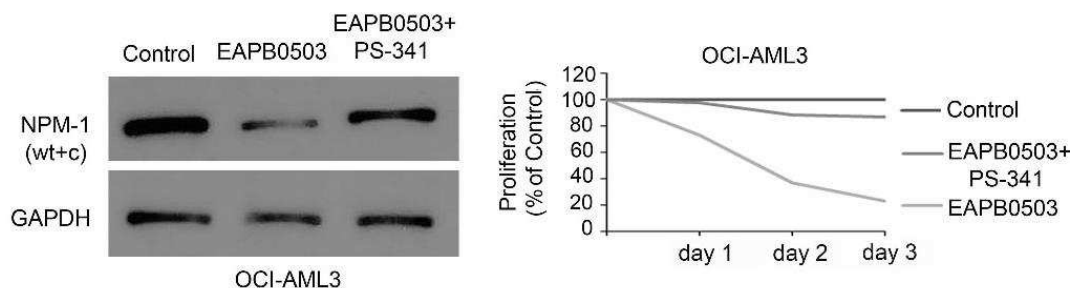
Figure 29. Quantitative real-time PCR analysis for *NPM-1* (*wt +c*) in OCI-AML3 and THP-1 cells.



Total RNA was extracted from control and EAPB0503-treated OCI-AML3 cells using the TRIzol extraction method. RNA concentration was then quantified using the Nanodrop spectrophotometer. cDNA was then prepared and Syber green quantitative real-time (RT)-PCR was performed using BIORAD machine.

Critically, both *NPM-1* downregulation and growth arrest were reversed following addition of the proteasome inhibitor PS-341 (Figure 30).

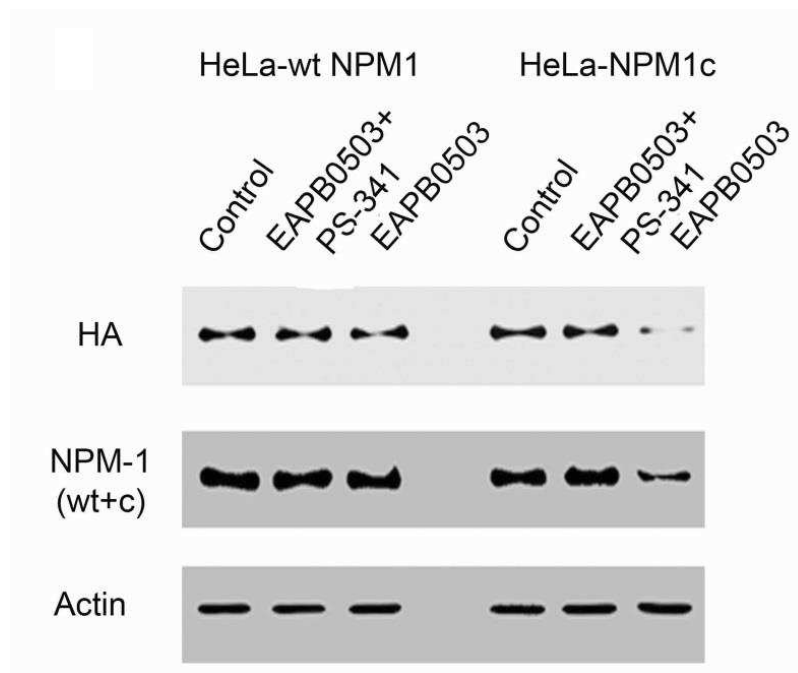
Figure 30. Western blot analysis for *NPM-1* (*wt +c*) in OCI-AML3 cells with and without the presence of the proteasome inhibitor PS-341.



Western blot analysis for *NPM-1* (*wt +c*) and actin in OCI-AML3 treated with 1 μ M of EAPB0503 alone, or in combination with 10 nM of the proteasome inhibitor PS-341 for 48 hours as indicated. Proliferation assay following treatment of OCI-AML3 with 1 μ M of EAPB0503 alone or in combination with 10 nM of PS-341 for 24, 48 and 72 hours. Cell growth is presented as (% of control) as indicated.

To eliminate any potential off target effect of the treatment, we treated HA tagged wt- NPM-1 or NPM-1c transfected HeLa cells with EAP0503 alone or combined with PS-341. Using an anti-HA antibody, our results show that the EAPB0503 proteasomal mediated-degradation is selective for NPM-1c, and was reversed upon adding PS-341 (Figure 31).

Figure 31. Western blot analysis for *NPM-1* (wt +c) in HA tagged wt- NPM-1 or NPM-1c transfected HeLa cells with and without the presence of the proteasome inhibitor PS-341.



Western blot analysis for HA, NPM-1 (wt+c) and actin in HeLa cells transfected with HA tagged wt *NPM-1* or *NPM-1c*, and treated with 1 μ M of EAPB0503 alone, or in combination with 10 nM of PS-341 for 48 hours as indicated.

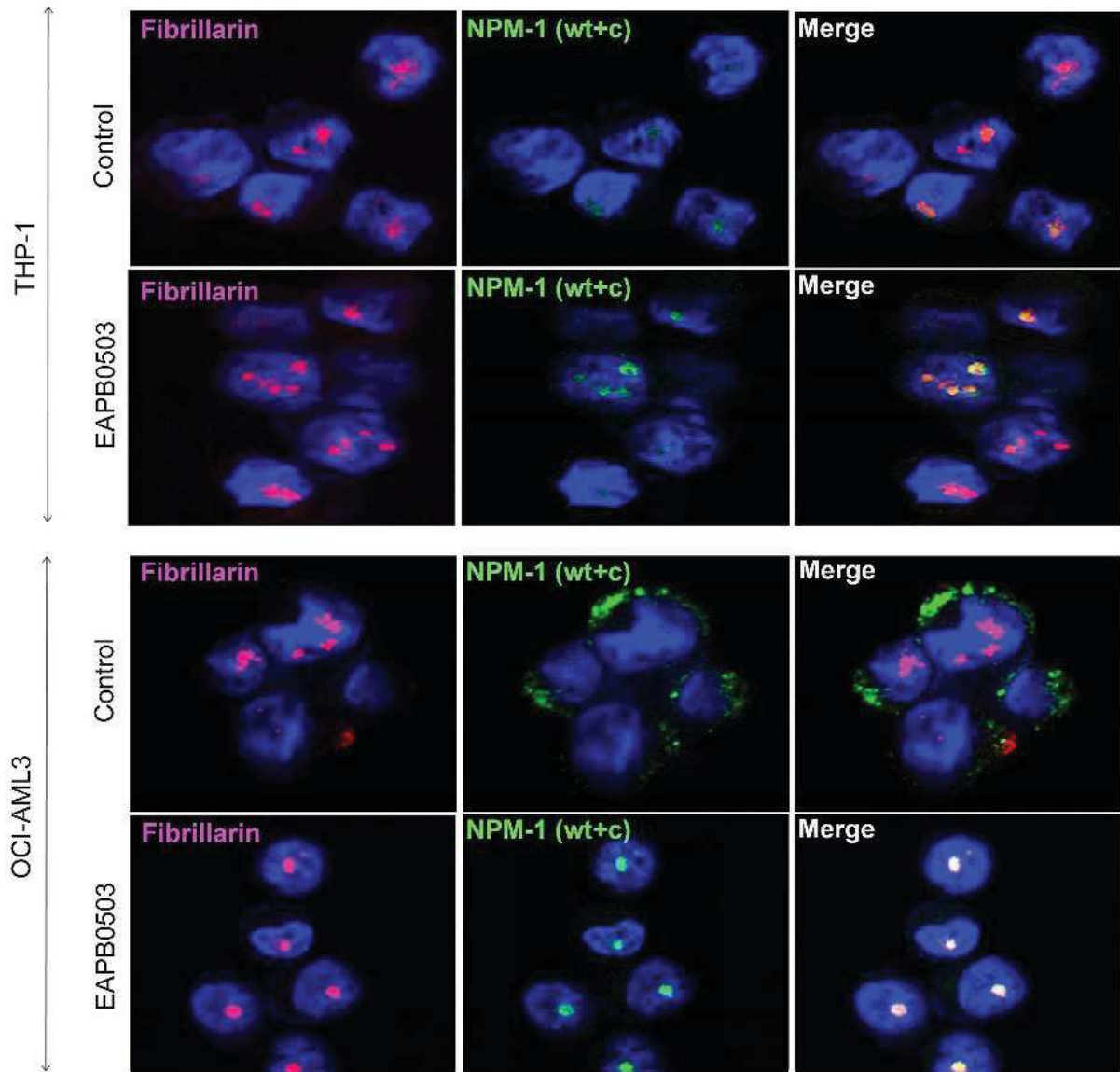
These results demonstrate that EAPB0503 induces proteasomal degradation of mutant *NPM-1* and strongly suggests that EAPB0503-induced growth arrest of AML cells with mutant *NPM-1* is secondary to oncoprotein degradation.

In order to understand the mechanism of downregulation of *NPM-1*, we looked at the transcript levels using primers specific for either the *NPM-1wt* or the mutated *NPM-1* mRNA, in control and in EAPB0503-treated cells. Transcript levels of both *wt* and mutant

NPM-1 were not affected by treatment (Figure 29) showing that *NPM-1* downregulation is not occurring at the transcriptional level, but rather at the protein level.

In *NPM-1* mutant AMLs, *NPM-1wt* oligomerizes with mutant *NPM-1* and is delocalized to the cytoplasm (Falini, Mecucci et al. 2005, Bolli, Nicoletti et al. 2007, Falini, Bolli et al. 2009) (Figure 31). Whereas treatment of *NPM-1wt* THP-1 cells with EAPB0503 did not affect the nucleolar localization of NPM-1 (Figure 32), treatment of OCI-AML3 with EAPB0503 restored the nucleolar localization of the remaining NPM-1 protein (Figure 32). This suggests that degradation of NPM-1c triggered by EAPB0503 releases *NPM-1wt* and thus corrects the defects in nucleolar organization.

Figure 32. Confocal microscopy analysis of nucleolar NPM-1 localization in OCI-AML3 or THP-1 cells after treatment.

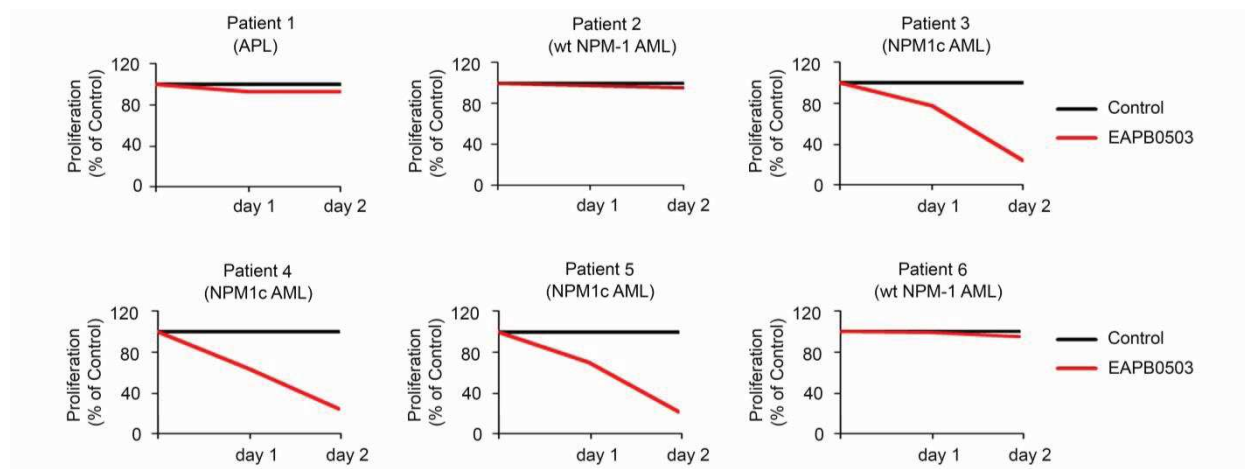


NPM-1 was stained with an antibody recognizing NPM-1 (wt +c) (green), nucleoli were stained with anti-Fibrillarin (red) and nuclei were stained with DAPI (blue). Images represent z-sections.

EAPB0503 selectively inhibits the proliferation, induces NPM-1c degradation and restores the wt-NPM-1 nucleolar localization in *ex-vivo* treated *NPM-1c* AML blasts

Primary blasts derived from the BM of six AML patients were treated with EAPB0503. Patient 1 had Acute promyelocytic leukemia (APL) with abnormal promyelocytic leukemia/retinoic acid receptor alpha (PML/RARA) rearrangement, patients 2 and 6 were AML patients with *wt-NPM-1*, and patients 3, 4 and 5 harbored an *NPM-1* mutation without *FLT-3 ITD*. Whereas leukemic cells derived from patients 1, 2 and 6 were not sensitive to EAPB0503 treatment, those derived from patients 3, 4 and 5 were highly sensitive and almost all died within 48h p.t. (Figure 33).

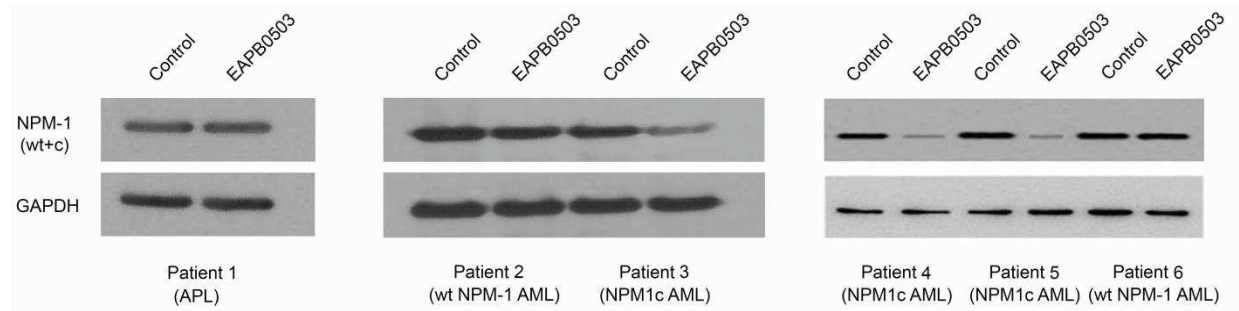
Figure 33. EAPB0503 inhibits the proliferation, induces degradation of NPM-1c and restores the nucleolar localization of wt-NPM-1 selectively in *ex-vivo* treated blasts derived from *NPM-1c* AML patients.



Proliferation of AML blasts after treatment for 24, 48 and 72 hours. Cell growth is represented as (% of control).

Moreover, EAPB0503 induced NPM1c selective degradation in patients 3, 4 and 5 (Figure 34).

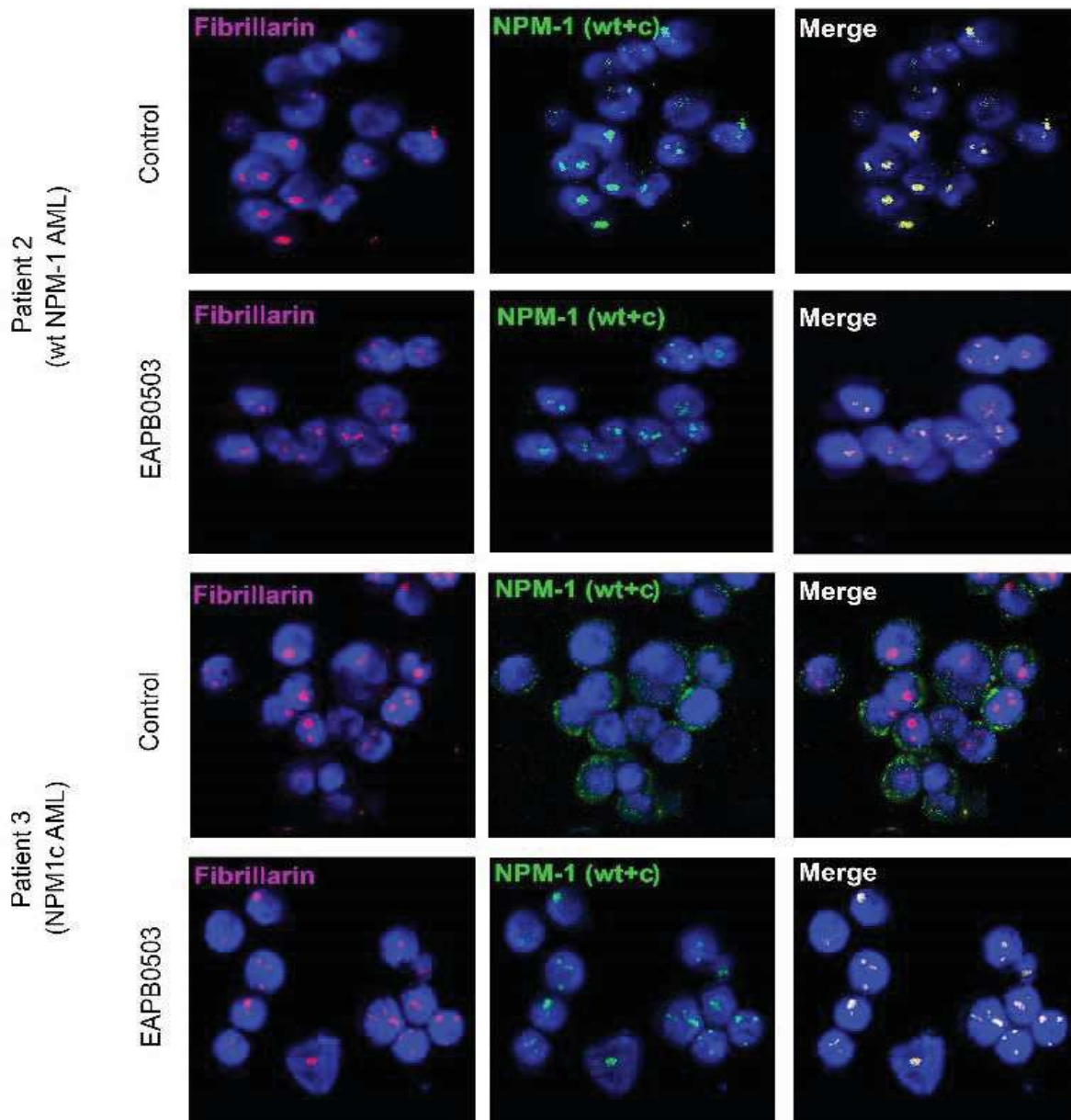
Figure 34. Western blot analysis for *NPM-1 (wt + c)* in control and treated cells derived from APL and AML patients.



Western blot analysis for NPM-1 (wt +c) and GAPDH in treated AML blasts as indicated above.

Furthermore, EAPB0503 restored the wt-NPM-1 nucleolar localization only in these patients (Figure 35). Collectively, EAPB0503 exerts its growth inhibition effect, induces NPM-1c degradation and corrects the wt-NPM-1 nucleolar localization, selectively in treated *NPM-1c* AML blasts *ex vivo*.

Figure 35. Confocal microscopy analysis of nucleolar NPM-1 localization in human blasts from 2 AML patients.



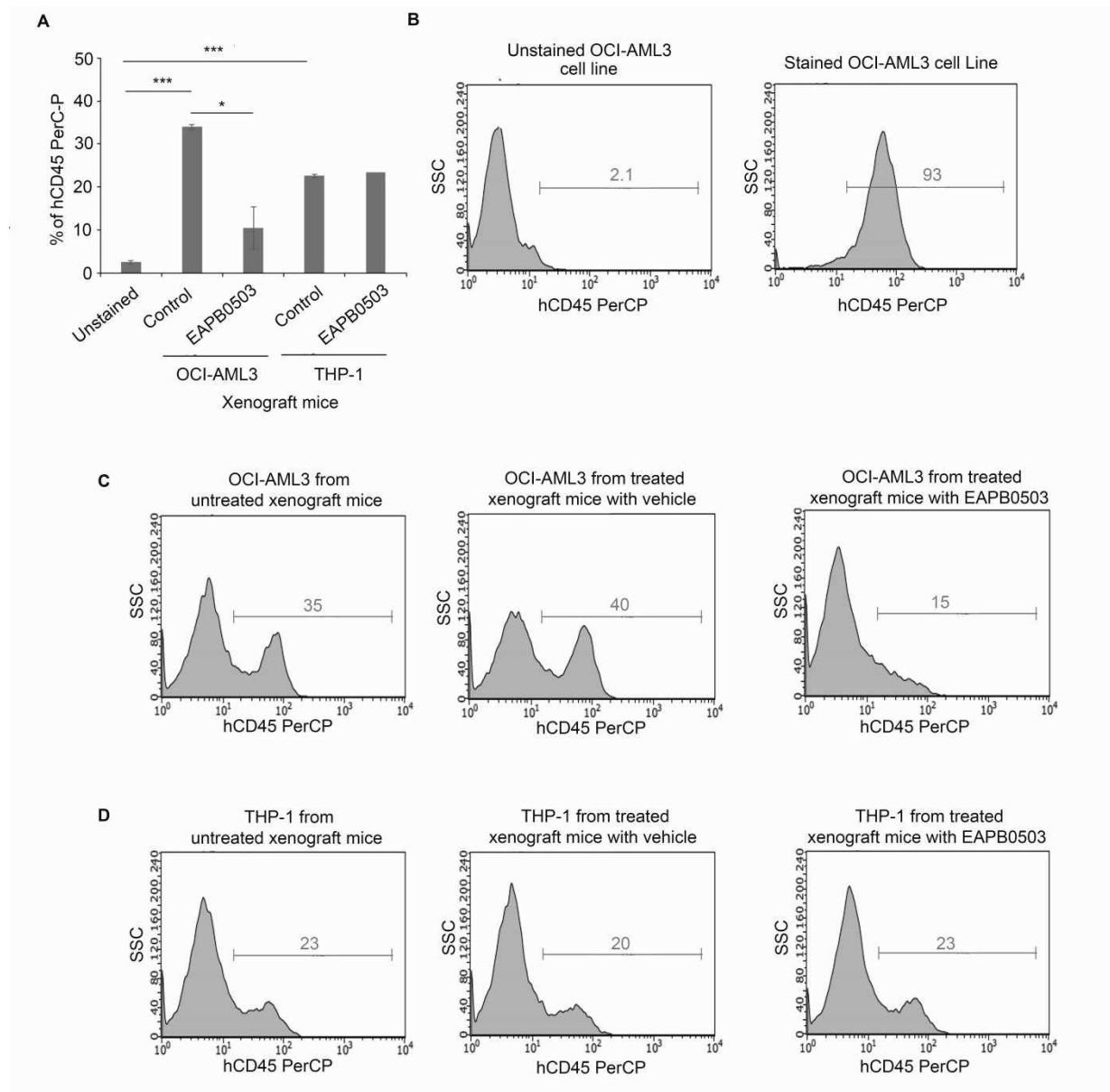
Confocal microscopy on patients' 2 and 3 derived blasts. NPM-1 (wt +c) was stained with an anti-NPM-1 (wt +c) antibody (green), nucleoli were stained with anti- Fibrillarin (red) and nuclei were stained with DAPI (blue). Images represent z-sections.

Collectively, EAPB0503 exerts its selective growth inhibition effect, NPM-1c degradation and correction of the NPM-1wt nucleolar localization, selectively on *NPM-1* mutant AML derived blasts *ex vivo*.

EAPB0503 selectively reduces leukemia bone marrow burden in OCI-AML3 xenograft mice

Several xenograft mouse models were generated.(Cesano, Hoxie et al. 1992, Quentmeier, Martelli et al. 2005) Furthermore, OCI-AML3 and THP-1 cells are known to express the hCD45 marker (Pfau, Walker et al. 2000, Quentmeier, Martelli et al. 2005). To assess the *in vivo* efficacy of EAPB0503, we injected NSG mice with OCI-AML3 or THP-1 cells. 5 days post AML cells injection, xenograft mice were treated intraperitoneally with EAPB0503 or its respective vehicle (DMSO/lipofundin) once daily, for 5 consecutive days a week, over a period of 2 weeks. At the end of week 3 post AML cells inoculation, femurs and tibias BM were flushed from untreated and vehicle or EAPB0503 treated mice. Human AML xenograft cells were stained with the human specific hCD45⁺ antibody and analyzed by flow cytometry. Our results show that OCI-AML3 BM burden was markedly reduced from 35% to 10% upon EAPB0503-treatment ($p < 0.05$) (Figures 36A, 36B) as compared to THP-1 burden that was not affected (22% in untreated versus 23% in EAPB0503 treated mice) (Figures 36C, 36D). These results indicate that EAPB0503 is a promising drug that selectively reduces *NPM-1c* AML BM burden in xenograft animals and warrant more pre-clinical then clinical investigation.

Figure 36. EAPB0503 selectively reduces leukemia bone marrow burden in OCI-AML3 xenograft NSG mice.



8 weeks old female NSG mice were injected with 1×10^6 OCI-AML3 or THP-1 cells intravenously. EAPB0503 or its vehicle were administered for 5 days a week over the period of two weeks intraperitoneally. At the end of week 3, bone marrow was harvested from femurs and tibias of xenograft mice then stained using the anti-hCD45 antibody. **A.** Histograms showing the hCD45 PerC-P percentage in xenograft animals. **B.** Unstained and stained OCI-AML3 cell line with hCD45 antibody. **C.** Representative histograms of stained untreated (left panel), treated with vehicle (middle panel) and EAPB0503 (right panel) OCI-AML3 xenograft mice **D.** Representative histograms of stained untreated (left panel), treated with vehicle (middle panel) and EAPB0503 (right panel) THP-1 xenograft mice.

4. PRISM Data on MTT

The concept of IC_{50} is pharmacologically very important. The IC_{50} is the concentration of a drug where the viability of cells is reduced by half. It is not that simple. When you actually want to be more accurate in determining these values, there are several complexities and ambiguities.

PRISM software provides a powerful combination of biostatistics that helps in finding an accurate IC_{50} based on the value R^2 that quantifies goodness of fit.

- When R^2 equals 0.0, the best-fit curve fits the data no better than a horizontal line going through the mean of all Y values. In this case, knowing X does not help you predict Y.
- When $R^2=1.0$, all points lie exactly on the curve with no scatter. If you know X you can calculate Y exactly.

Results show that IC_{50} is around 4.2 with a relatively high goodness value (R^2) that is almost equal to 1 ($R^2=0.9962$) (Figure 37). Cells were treated with increasing concentrations of EAPB0503 for 48 hours.

Figure 37. MTT assay on OCI-AML3 cells using PRISM software.

Change		New Analysis	Change Params.	Nonlinear reg. (fit)	View: Table of results
		X Labels		A	
				EAPB0503	
		X		Y	
1	Equation 1				
2	Variables				
3	BOTTOM	102.0			
4	TOP	37.09			
5	LOGEC50	-6.384			
6	EC50	4.133e-007			
7	Std. Error				
8	BOTTOM	3.778			
9	TOP	1.643			
10	LOGEC50	0.08284			
11	95% Confidence Intervals				
12	BOTTOM	85.76 to 118.3			
13	TOP	30.02 to 44.16			
14	LOGEC50	-6.740 to -6.027			
15	EC50	1.819e-007 to 9.391e-007			
16	Goodness of Fit				
17	Degrees of Freedom		2		
18	R ²		0.9962		
19	Absolute Sum of Squares		6.264		
20	Sy.x		1.770		
21	Data				
22	Number of X values		5		
23	Number of Y replicates		1		
24	Total number of values		5		

5. Research paper (*Cancer Journal*)

Imidazoquinoxaline Derivative EAPB0503: A Promising Drug Targeting Mutant Nucleophosmin 1 in Acute Myeloid Leukemia

Ali I. Nabbouh, MS^{1,2}; Rita S. Hleihel, MS^{1,3}; Jessica L. Saliba, PhD⁴; Martin M. Karam, MS^{1,5}; Maguy H. Hamie, MS^{1,5}; Hsin-Chieh J.M. Wu, PhD⁶; Caroline P. Berthier⁶; Nadim M. Tawil, MS⁷; Pierre-Antoine A. Bonnet, PhD²; Carine Deleuze-Masquefa, PhD²; and Hiba A. El Hajj, PhD^{1,5}

BACKGROUND: Nucleophosmin 1 (NPM1) is a nucleocytoplasmic shuttling protein mainly localized in the nucleolus. *NPM1* is frequently mutated in acute myeloid leukemia (AML). NPM1c oligomerizes with wild-type nucleophosmin 1 (wt-NPM1), and this leads to its continuous cytoplasmic delocalization and contributes to leukemogenesis. Recent studies have shown that Cytoplasmic NPM1 (NPM1c) degradation leads to growth arrest and apoptosis of *NPM1c* AML cells and corrects wt-NPM1 normal nucleolar localization. **METHODS:** AML cells expressing wt-NPM1 or NPM1c or transfected with *wt-NPM1* or *NPM1c* as well as *wt-NPM1* and *NPM1c* AML xenograft mice were used. Cell growth was assessed with trypan blue or a CellTiter 96 proliferation kit. The cell cycle was studied with a propidium iodide (PI) assay. Caspase-mediated intrinsic apoptosis was assessed with annexin V/PI, the mitochondrial membrane potential, and poly(adenosine diphosphate ribose) polymerase cleavage. The expression of NPM1, p53, phosphorylated p53, and p21 was analyzed via immunoblotting. Localization was performed with confocal microscopy. The leukemia burden was evaluated by flow cytometry with an anti-human CD45 antibody. **RESULTS:** The imidazoquinoxaline 1-(3-methoxyphenyl)-*N*-methylimidazo[1,2-*a*]quinoxalin-4-amine (EAPB0503) induced selective proteasome-mediated degradation of NPM1c, restored wt-NPM1 nucleolar localization in *NPM1c* AML cells, and thus yielded selective growth arrest and apoptosis. Introducing *NPM1c* to cells normally harboring *wt-NPM1* sensitized them to EAPB0503 and led to their growth arrest. Moreover, EAPB0503 selectively reduced the leukemia burden in *NPM1c* AML xenograft mice. **CONCLUSIONS:** These findings further reinforce the idea of targeting the NPM1c oncoprotein to eradicate leukemic cells and warrant a broader preclinical evaluation and then a clinical evaluation of this promising drug. *Cancer* 2017;000:000-000. © 2017 American Cancer Society.

KEYWORDS: acute myeloid leukemia, apoptosis, 1-(3-methoxyphenyl)-*N*-methylimidazo[1,2-*a*]quinoxalin-4-amine (EAPB0503), nucleophosmin 1, xenograft mice.

INTRODUCTION

Acute myeloid leukemia (AML) is a complex, heterogeneous blood malignancy in which a failure to differentiate and an overproliferation of undifferentiated myeloid precursors result in impaired hematopoiesis and bone marrow (BM) failure. AML is associated with a highly variable prognosis and a high mortality rate, with overall survival exceeding 2 years for only 20% of elderly patients and 5 years for less than 50% of adult patients.¹

The prognosis of AML is mostly dependent on somatic genetic alterations used to classify the risk as favorable, intermediate, or unfavorable.² In AML patients with a normal karyotype, the most important genetic mutations influencing both the prognosis and the treatment strategies are mutations in nucleophosmin 1 (*NPM1*) and FMS-like tyrosine kinase 3 (*FLT-3*) internal tandem duplication.³ Recently, more heterogeneous genomic categories for AML have been reported.⁴

Corresponding author: Hiba A. El Hajj, PhD, Department of Internal Medicine, American University of Beirut, P.O. Box 113-6044, Beirut, Lebanon; FAX: (011) 961 1 343 450; he21@aub.edu.lb

¹Department of Internal Medicine, American University of Beirut, Beirut, Lebanon; ²Max Mousseron Institute of Biomolecules, Faculty of Pharmacy, Montpellier University, Montpellier, France; ³Department of Cell Biology, Anatomy, and Physiological Sciences, American University of Beirut, Beirut, Lebanon; ⁴Department of Biology, Faculty of Science, Lebanese University, Beirut, Lebanon; ⁵Department of Experimental Pathology, Microbiology, and Immunology, American University of Beirut, Beirut, Lebanon; ⁶National Institute of Health and Medical Research Unit 944, Collège de France, Paris, France; ⁷Department of Experimental Medicine, McGill University, Montreal, Canada.

The first 2 authors contributed equally to this article.

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Additional supporting information may be found in the online version of this article.

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NPM1 is an essential gene⁵ encoding a phosphoprotein⁶ continuously shuttling between the nucleus, nucleolus, and cytoplasm but mainly residing in the nucleolus.^{7,8} *NPM1* has many functions, including p14^{Arf} stabilization, ribosomal biogenesis regulation, centrosomal duplication control, and p53 activation in response to stress stimuli.^{5,6,9} In AML, *NPM1* mutations account for approximately one-third of patients, and this makes it one of the most frequently mutated genes.^{6,10} These mutations lead to the creation of a *de novo* nuclear export signal,^{6,10,11} which results in cytoplasmic accumulation of NPM1c, along with wild-type nucleophosmin 1 (wt-NPM1) and thus leukemogenesis in these AML patients.¹⁰

Despite all the advances in genetic and epigenetic changes in AML, there is still little progress in the treatment of the disease. Although complete remission is reached by almost 70% of patients with standard induction chemotherapy, refractory disease is common, and relapse represents the major cause of treatment failure.¹² Stem cell transplantation remains the best chance for long-term survival but is associated with several complications.¹³ Therefore, new therapeutic approaches, specifically ones directly targeting the products of AML genetic alterations, are needed.

In *NPM1c* AML, degradation of the NPM1c oncoprotein leads to leukemic cell growth arrest and apoptosis.¹⁴⁻¹⁶ We and others have recently shown that arsenic trioxide and retinoic acid selectively induce NPM1c proteasomal degradation and thus lead to apoptosis in *NPM1c* AML cells.^{15,16} This combined treatment restores NPM1 nucleolar localization *ex vivo* and *in vivo*. However, although the clearance of AML blasts was observed in a few treated patients, no cure was achieved, likely because of the complexity and status of the disease burden. This underlies the need for novel therapies to improve treatment outcomes.

Imiquimod is a toll-like receptor 7 immunomodulator^{17,18} used to treat certain skin cancers¹⁹ and genital warts.²⁰ Imiquimod analogues, called imidazoquinoxalines, have been synthesized²¹; among them, 1-(2-phenylethyl)-*N*-methylimidazo[1,2-*a*]quinoxalin-4-amine (EAPB0203) and 1-(3-methoxyphenyl)-*N*-methylimidazo[1,2-*a*]quinoxalin-4-amine (EAPB0503) have been reported with promising antitumor activity.^{22,23} Indeed, EAPB0203 displayed pronouncedly higher *in vitro* potency against melanoma and adult T-cell leukemia cells in comparison with imiquimod.^{23,24} Later, EAPB0503 showed 10-fold higher cytotoxicity than EAPB0203 against melanoma cells.²⁵ More recently, EAPB0503

showed a potent apoptotic effect in chronic myeloid leukemia cells through BCR-ABL degradation.²⁶

Here we demonstrate that EAPB0503 induces NPM1c proteasomal degradation selectively in *NPM1c* AML cells and leads to their apoptosis. Importantly, introducing *NPM1c* to *wt-NPM1*-harboring cells sensitizes them to EAPB0503. Moreover, EAPB0503 treatment restores wt-NPM1 nucleolar localization *in vitro* and also *in ex vivo* treated blasts and selectively reduces the leukemia burden in *NPM1c* AML xenograft mice. These findings expand the antileukemic use of EAPB0503, reinforce the idea of targeting oncoprotein degradation to kill leukemic cells, and warrant a broader preclinical evaluation and then a clinical evaluation of this promising drug.

MATERIALS AND METHODS

Cell Lines

KG-1 α , ML-2, and THP-1 cell lines (from F. Mazurier) and IMS-M2 (from H. de Th ) were grown in Roswell Park Memorial Institute 1640 medium. OCI-AML3 cells (from D. Bouscary) were grown in minimum essential medium α . Cells were seeded at a concentration of 2×10^5 /mL. EAPB0203 or EAPB0503 was used at 0.1 to 5 μ M, the caspase inhibitor Z-Val-Ala-DL-Asp(OMe)-fluoromethylketone (zVAD) (Bachem Bioscience) was used at 50 μ M, and the proteasome inhibitor PS-341 was used at 10 nM.¹⁵ Cell growth was assessed with trypan blue or a CellTiter 96 proliferation kit (Promega).

Primary AML cells from patients' BM were extracted as described by El Hajj et al¹⁵ after approval by the institutional review board at the American University of Beirut and after the patients had consented according to the Declaration of Helsinki.

Drugs

The synthesis of EAPB0203 and EAPB0503 was performed as described by Deleuze-Masquefa et al.^{21,22} Further optimization of EAPB0503 synthesis was achieved with microwave-assisted chemistry.²⁷

Generation of Cells Expressing wt-NPM1 or NPM1c

Green fluorescent protein (*GFP*) *wt-NPM1* or *NPM1c* inserts were amplified and ligated into a pBybe lentiviral vector by the EcoRI site. Stable OCI-AML2 expressing wt-NPM1 or NPM1c was generated by lentiviral transduction followed by blasticidin selection. GFP-positive cells were sorted with the FACSAria Special Order Research Product (Becton Dickinson) and grown in minimum essential medium α before the cell growth assessment.

HeLa cells were transfected with pcDNA hemagglutinin (HA) expressing *wt-NPM1* or *NPM1c* (from G. Tell)²⁸ with Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations and were grown in Dulbecco's modified Eagle's medium.

Xenograft Animal Studies

NOD/Shi-*scid* *IL2r γ* ^{-/-} (NSG) mice were obtained from Jackson Laboratories (United States). Mouse protocols were approved by the institutional animal care and utilization committee of the American University of Beirut. OCI-AML3 or THP-1 cells (1×10^6) were injected into the tail vein of 8-week-old females (5 mice per group). On day 5 after the AML injection, the mice were treated with EAPB0503 (15 mg/kg) for 5 days a week over a period of 2 weeks. EAPB0503 was dissolved in dimethyl sulfoxide and diluted in an equal volume of lipofundin (vehicle) before its intraperitoneal administration to the mice.^{24,29}

Flow Cytometry

Cell cycle analysis

Propidium iodide (PI) staining was used to assess the cell cycle as described by El Hajj et al.¹⁵

Annexin V staining

An annexin V–fluorescein isothiocyanate kit (BD Pharmingen) was used to assess phosphatidylserine exposure. Cells were treated with 1 μ M EAPB0503 for 24 hours before annexin V/PI labeling and flow cytometry analysis.

Mitochondrial membrane potential (MMP)

The MMP was assessed by a cell's ability to retain rhodamine 123 (Sigma-Aldrich), as described by Saliba et al.²⁶

A Becton Dickinson FACS instrument was used; 10,000 events per condition were acquired, and FlowJo software (FlowJo LLC) was used for the analysis of the results.

Human CD45 staining

BM from the femurs and tibias of euthanized animals was flushed at the end of week 3 after AML inoculation. Cell surface staining was performed on 100 μ L of a sample with 20 μ L of an anti-human CD45 Peridinin Chlorophyll Protein (PerCP) conjugated antibody (345809; Becton Dickinson). After incubation for 15 minutes in the dark, erythrocytes were lysed with 1 mL of an FACS lysis solution (Becton Dickinson). Labeled samples were washed twice and analyzed on a Guava flow cytometer.

Immunoblot Analysis

After 48 hours of treatment with EAPB0203 or EAPB0503, proteins were probed with poly(adenosine diphosphate ribose) polymerase (PARP), p53, p21, HA (Santa Cruz), phosphorylated p53 (Biolabs), or NPM1 (Abcam) before incubation with the monoclonal horseradish peroxidase–conjugated secondary antibodies. The loading control was performed via probing with the mouse horseradish peroxidase–conjugated glyceraldehyde 3-phosphate dehydrogenase antibody (Abnova) or β -actin (Abcam). Immunoblots were detected with a luminol detection kit (Santa Cruz), and images were captured with the X-OMAT or BioRad ChemiDoc MP system.

Immunofluorescence Microscopy

AML cells or patients' blasts were spun down onto glass slides, fixed, and permeabilized with ice-cold methanol for 30 minutes. Immunostaining was performed with a monoclonal antibody against anti-B23 NPM1 (Santa Cruz) and a polyclonal antibody against the nucleolar marker fibrillarin (Abcam). Primary antibodies were revealed by Alexa Fluor 488– or Fluor 594–labeled secondary antibodies (Santa Cruz). Images were acquired with a Zeiss LSM 710 laser scanning microscope operated with Zen 2009 software (Carl Zeiss).

Statistical Analysis

Data are reported as averages and standard deviations. Statistical analyses were performed with the Student *t* test; a *P* value less than .05 was considered significant.

RESULTS

EAPB0203 and EAPB0503 Induce Growth Arrest in *NPM1c* AML Cells

We used 3 *wt-NPM1* cell lines (THP-1, KG-1 α , and MOLM-13) and the 2 available *NPM1c* AML cell lines (OCI-AML3 and IMS-M2) to test for EAPB0203 and EAPB0503 effects on cell growth and viability. We tested a range of drug concentrations (0.1–5 μ M) and assessed cell growth for 5 days after treatment. Both treatments resulted in pronounced time-dependent growth inhibition of OCI-AML3 cells (Fig. 1A,B). EAPB0203 at 5 μ M resulted in significant OCI-AML3 growth inhibition (*P* < .05), which started 72 hours after treatment. Strikingly, EAPB0503 was more potent and at 0.1 μ M resulted in significant growth inhibition, which started 96 hours after treatment (*P* < .001). Similarly significant results were obtained for both OCI-AML3 and IMS-M2: a concentration of 0.5 μ M induced growth inhibition starting 72 hours after treatment (*P* < .001), and concentrations

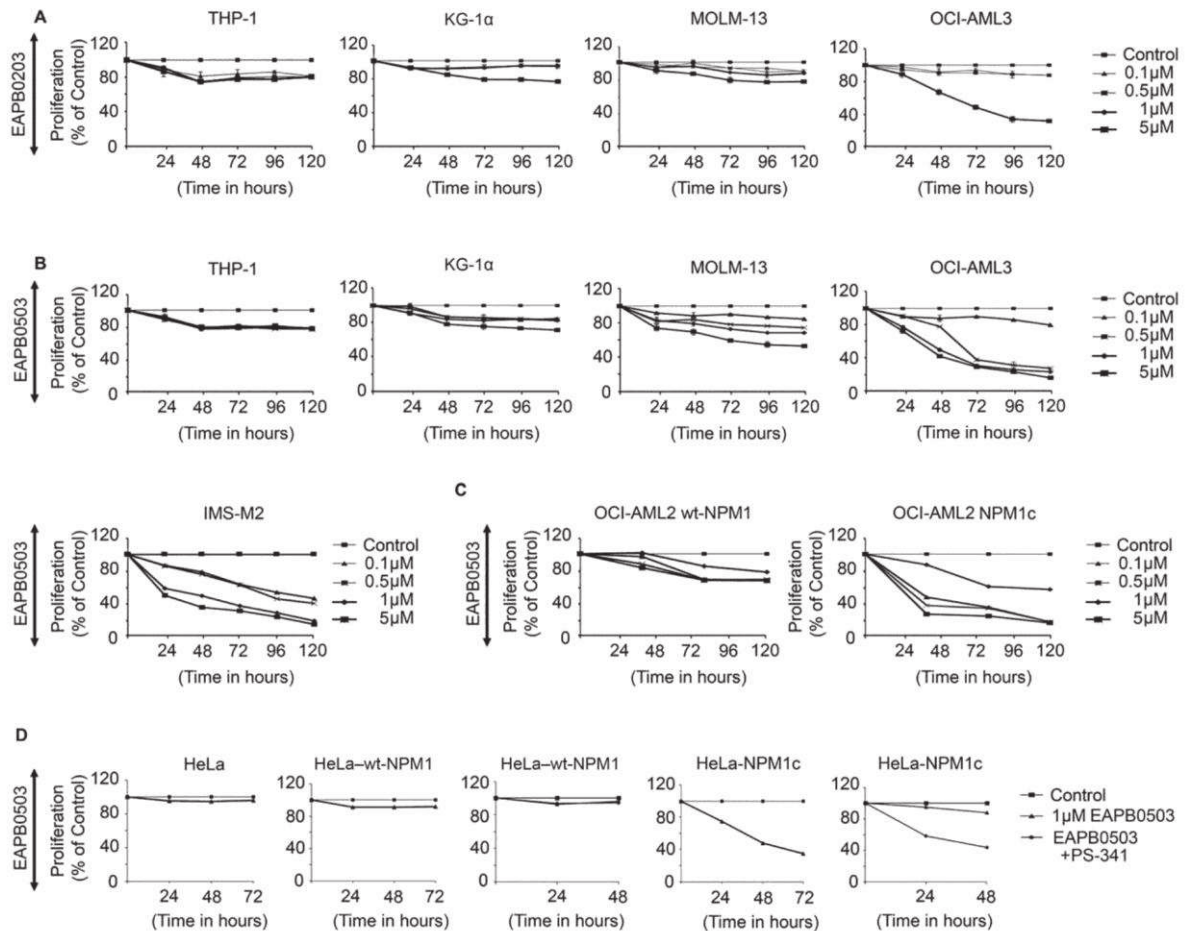


Figure 1. EAPB0503 induces selective growth inhibition in *NPM1c* AML cells. AML cell lines with normal *NPM1* (THP-1, KG-1α, and MOLM-13) and *NPM1c* (OCI-AML3 and IMS-M2) were treated with increasing concentrations (0.1–5 μM) of (A) EAPB0203 and (B) EAPB0503 for 24, 48, 72, 96, and 120 hours. (C) Stably transfected OCI-AML2 with green fluorescent protein *wt-NPM1* or *NPM1c* was treated with increasing concentrations (0.1–5 μM) of EAPB0503 for 24, 48, 72, 96, and 120 hours. (D) HeLa cells transfected with hemagglutinin-tagged *wt-NPM1* or *NPM1c* were treated with 1 μM EAPB0503 alone or in combination with 10 nM PS-341 for 24, 48, and 72 hours as indicated. Cell growth (percentage of the control) was assayed in triplicate. The results represent the average of at least 3 independent experiments. AML indicates acute myeloid leukemia; EAPB0203, 1-(2-phenylethyl)-*N*-methylimidazo[1,2-*a*]quinoxalin-4-amine; EAPB0503, 1-(3-methoxyphenyl)-*N*-methylimidazo[1,2-*a*]quinoxalin-4-amine; *NPM1*, nucleophosmin 1; *wt-NPM1*, wild-type nucleophosmin 1; Cytoplasmic *NPM1* (*NPM1c*) Hemagglutinin (HA)-tagged confirmed green fluorescent protein (GFP)-tagged *wt-NPM1* or *NPM1c*.

of 1 and 5 μM induced the same inhibitory effect 24 hours after treatment ($P < .05$ and $P < .001$, respectively; Fig. 1B). Importantly, a median inhibitory concentration of 1 μM in OCI-AML3 and IMS-M2 cells was achieved 2 days after treatment with EAPB0503 ($P < .05$ and $P < .001$, respectively), whereas a concentration of 5 μM was achieved after treatment with EAPB0203 in OCI-AML3 (Fig. 1A,B). This more potent effect of EAPB0503 versus EAPB0203 is in line with previously reported results.²⁶ THP-1 and KG-1α cells were minimally sensitive to the compounds, with only approximately 20% growth inhibition even 5 days after treatment (Fig. 1A,B). MOLM-13 cells were also minimally sensitive to

EAPB0203 but displayed approximately 50% growth inhibition 72 hours after treatment with EAPB0503 (Fig. 1A,B). This percentage did not become more pronounced even 5 days after treatment, and the only significant result was obtained with concentrations of 1 and 5 μM, 120 and 72 hours after treatment, respectively ($P < .05$; Fig. 1B).

Introduction of *NPM1c* Into *wt-NPM1*-Expressing Cells Sensitizes Them to EAPB0503

To examine whether the growth inhibition solely observed in *NPM1c* cell lines was due to *NPM1* mutations, we introduced *NPM1c* to *wt-NPM1*-expressing cells and checked for their sensitivity to EAPB0503. We used the

wt-NPM1-expressing AML cell line (OCI-AML2) and generated by lentiviral transduction and then blasticidin selection cells stably expressing either GFP-tagged wt-NPM1 or NPM1c. GFP-positive cells were sorted, and a range of EAPB0503 concentrations (0.1–5 μ M) were tested to assess cell growth more than 5 days after treatment. Interestingly, stable expression of NPM1c in OCI-AML2 resulted in significantly pronounced growth inhibition at 0.1 μ M that started 72 hours after treatment and at 0.5, 1, and 5 μ M that started 48 hours after treatment ($P < .05$; Fig. 1C). A minimal effect was observed in wt-NPM1 OCI-AML2: maximum growth inhibition of 30% (nonsignificant) was obtained 48 hours after treatment with concentrations of 0.5, 1, and 5 μ M (Fig. 1C). Similar results were obtained with HeLa cells: a concentration of 1 μ M induced growth arrest starting 48 hours after treatment in HA *NPM1c*-transfected cells ($P < .001$) but not wt-NPM1-transfected cells (Fig. 1D). This growth inhibition was reversed upon the addition of PS-341 only in NPM1c-expressing cells both 24 and 48 hours after treatment ($P < .05$; Fig. 1D). Our results strongly suggest that introducing *NPM1c* into cells harboring wt-NPM1 sensitizes them to EAPB0503. Because of its potency, especially in *NPM1c* AML cells, only EAPB0503 was adopted at its median inhibitory concentration of 1 μ M for the remainder of the study.

EAPB0503 Induces Massive Apoptosis in *NPM1c* AML Cells

To examine the mechanisms dictating growth inhibition and cell death, a cell cycle analysis was performed 48 hours after treatment with 1 μ M EAPB0503. A sharp increase in the pre-G₀ cell percentage, which reached more than 80%, was obtained upon the treatment of OCI-AML3 with EAPB0503. Minimal effect was observed in the wt-NPM1 cells (THP-1, KG-1 α , and MOLM-13; Fig. 2A and Supporting Fig. 1A [see online supporting information]). The cell cycle distribution showed no major variation in all the tested AML cells untreated or treated with EAPB0503 (Fig. 2B and Supporting Fig. 1A [see online supporting information]), and this shows that the drug is mostly inducing pre-G₀ accumulation in *NPM1c* AML without affecting the other cell cycle phases.

To confirm the apoptosis, annexin V/PI labeling was performed, and a significant increase of 40% in annexin V positivity was observed only in OCI-AML3 cells treated with 1 μ M EAPB0503 for 24 hours ($P < .005$; Fig. 2C and Supporting Fig. 1B [see online supporting information]). In contrast, all wt-NPM1 cells remained virtually annexin V-negative upon treatment with the drug (Fig. 2C and Supporting Fig. 1B [see online supporting information]).

EAPB0503-Induced Apoptosis in *NPM1c* AML Cells Involves the Dissipation of MMP and Caspase Activation

The intrinsic apoptotic cascade is characterized by many steps, the earliest of which is the disruption of the MMP.³⁰ Because EAPB0503 induces apoptosis in *NPM1c* AML cells, we measured MMP in untreated cells or 2 days after treatment with EAPB0503. Treated OCI-AML3 cells failed to retain the rhodamine 123 dye inside their mitochondria (Fig. 2D and Supporting Fig. 1C [see online supporting information]). Conversely, all wt-NPM1 AML cells showed no loss of MMP up to 48 hours after treatment (Fig. 2D and Supporting Fig. 1C [see online supporting information]).

To study the effect of MMP dissipation in EAPB0503-treated AML cells on the caspase cascade, we examined PARP cleavage. The treatment of OCI-AML3 for 48 hours with EAPB0503 but not with EAPB0203 led to PARP cleavage into its death-associated fragment (Fig. 2E); this occurred to a much lesser extent in the wt-NPM1 AML cells treated with either drug (Fig. 2E). Interestingly, the cotreatment of cells with the general caspase inhibitor zVAD and EAPB0503 reversed EAPB0503 growth-induced inhibition in OCI-AML3 (Fig. 2F), whereas no effect was observed in wt-NPM1 cells (THP-1 and MOLM-13; Fig. 2F). Altogether, our results indicate that the selective growth arrest obtained in *NPM1c* AML with EAPB0503 involves caspase activation.

EAPB0503 Treatment Activates p53 Signaling in *NPM1c* AML Cells

To determine whether the EAPB0503-associated growth inhibition and apoptosis were p53-mediated, p53 protein levels were monitored 48 hours after treatment with 1 μ M EAPB0203 or EAPB0503, and the results were compared with untreated controls. EAPB0503 induced substantial upregulation of total p53 protein levels and the p53 phosphorylated form exclusively in the *NPM1c* OCI-AML3 cell line (Fig. 2G), whereas no effect was observed upon the treatment of these cells with EAPB0203 (Fig. 2G). Accordingly, p21 protein levels were upregulated only in EAPB0503-treated OCI-AML3 (Fig. 2G). Because p53 is mutated in both THP-1 and KG-1 α cell lines,³¹ we tested p53 only in the wt-NPM1 MOLM-13 cell line and found that p53, phosphorylated p53, and p21 protein levels remained unchanged upon treatment with either drug (Fig. 2G). Altogether, these results show that EAPB0503 is a potent inducer of apoptosis exclusively in *NPM1c* AML cells.

EAPB0503 Induces NPM1c Proteasomal Degradation and Restores wt-NPM1 Nucleolar Localization in NPM1c AML Cells

Given the selective activity of EAPB0503 in *NPM1c* AML cells, we examined its effect on NPM1c oncoprotein

degradation. Although no effect of EAPB0203 or EAPB0503 on NPM1 expression was obtained in THP-1, MOLM-13, or KG-1 α cells (Fig. 3A), EAPB0503 but not EAPB0203 triggered NPM1 downregulation in OCI-AML3 cells (Fig. 3B), and this suggests that NPM1c is the

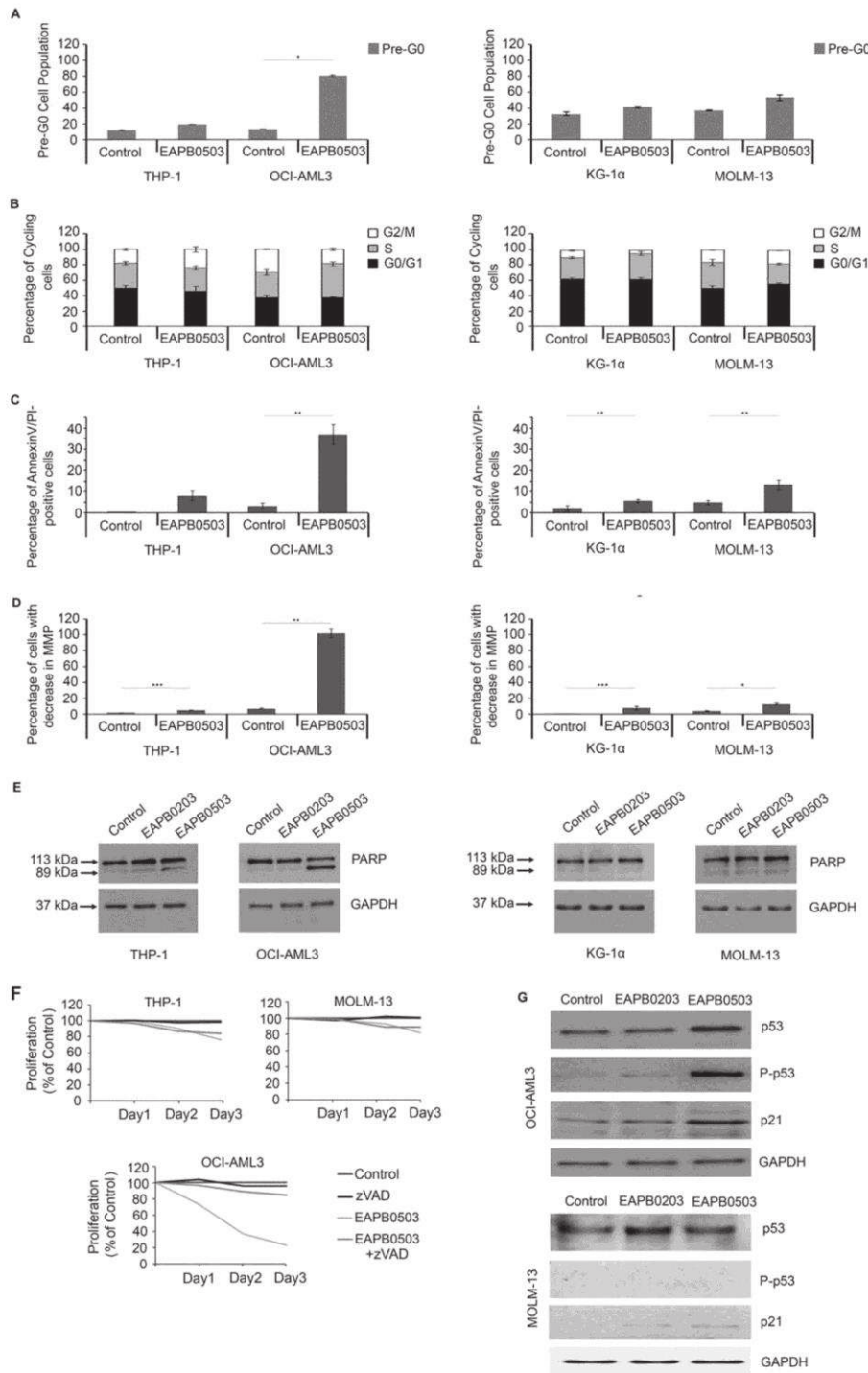


Figure 2.

primary target of EAPB0503. Critically, adding the proteasome inhibitor PS-341 reversed both NPM1 downregulation and growth arrest (Fig. 3C) specifically in OCI-AML3 (Supporting Fig. 2 [see online supporting information]). To eliminate any potential off-target effect of the treatment, we treated HA-tagged, *wt-NPM1*- or *NPM1c*-transfected HeLa cells with EAP0503 alone or in combination with PS-341. With an anti-HA antibody, our results showed that EAPB0503 proteasome-mediated degradation was selective for NPM1c and was reversed upon the addition of PS-341 (Fig. 3D). Using primers specific for either *wt-NPM1* or *NPM1c* messenger RNA, we found that neither transcript level was affected in EAPB0503-treated cells (Supporting Fig. 3 [see online supporting information]), and this shows that NPM1 downregulation occurs at the protein level. Collectively, these results strongly suggest that EAPB0503-treated *NPM1c* AML cells are secondary to oncoprotein degradation.

In *NPM1c* AML, *wt-NPM1* oligomerized with NPM1c and was delocalized to the cytoplasm (Fig. 3E),^{6,10,11} whereas the treatment of THP-1 cells with EAPB0503 did not affect NPM1 nucleolar localization (Fig. 3E), EAPB0503 treatment of OCI-AML3 restored the nucleolar localization of the remaining NPM1 protein (Fig. 3E). This suggests that EAPB0503-triggered degradation of NPM1c releases *wt-NPM1* and thus corrects the nucleolar organization defect.

EAPB0503 Selectively Inhibits Proliferation, Induces *NPM1c* Degradation, and Restores *wt-NPM1* Nucleolar Localization in Ex Vivo Treated *NPM1c* AML Blasts

Primary blasts derived from the BM of 6 AML patients were treated with EAPB0503. Patient 1 had acute promyelocytic leukemia with PML/RARA rearrangement, patients

2 and 6 were AML patients with *wt-NPM1*, and patients 3 to 5 harbored an *NPM1* mutation without *FLT-3* internal tandem duplication. Although leukemic cells derived from patients 1, 2, and 6 were not sensitive to EAPB0503 treatment, those derived from patients 3 to 5 were highly sensitive, and almost all died within the first 48 hours after treatment (Fig. 4A). Moreover, EAPB0503 induced NPM1c selective degradation in patients 3 to 5 (Fig. 4B) and restored the *wt-NPM1* nucleolar localization only in those patients (Fig. 4C). Collectively, EAPB0503 exerts its growth-inhibition effect, induces NPM1c degradation, and corrects the *wt-NPM1* nucleolar localization selectively in treated *NPM1c* AML blasts ex vivo.

EAPB0503 Selectively Reduces the Leukemia BM Burden in OCI-AML3 Xenograft Mice

Several xenograft mouse models have been generated.^{32,33} Furthermore, OCI-AML3 and THP-1 cells are known to express the hCD45 marker.^{33,34} To assess the in vivo efficacy of EAPB0503, we injected NSG mice with OCI-AML3 or THP-1 cells. Five days after the AML cell injection, xenograft mice were treated intraperitoneally with EAPB0503 or its respective vehicle (dimethyl sulfoxide/lipofundin) once daily for 5 consecutive days a week over a period of 2 weeks. At the end of week 3 after the AML cell inoculation, BM was flushed from the femurs and tibias of untreated mice and vehicle- or EAPB0503-treated mice. Human AML xenograft cells were stained with the human-specific hCD45⁺ antibody and analyzed with flow cytometry. Our results show that the OCI-AML3 BM burden was markedly reduced from 34% to 10% upon EAPB0503 treatment ($P < .05$; Fig. 5A,B), whereas the THP-1 burden was not affected (22% for untreated mice vs 23% for EAPB0503-treated mice; Fig. 5B,C). These results indicate that EAPB0503 is a promising drug that selectively

Figure 2. EAPB0503 induces caspase-mediated apoptosis in *NPM1c* AML cells. (A) Pre-G₀ cell population after PI staining upon the treatment of AML cell lines with the median inhibitory concentration dose (1 μM) of EAPB0503 for 48 hours. (B) Percentage of cycling cell populations after PI staining upon the treatment of AML cells for 48 hours as described previously. Histograms represent the relative distributions of nonapoptotic cells between the G₀/G₁, S, and G₂/M phases. (C) Annexin V staining of AML cells treated for 48 hours as described previously. (D) MMP assay. After the treatment with AML cells as described previously and rhodamine 123 staining, rhodamine 123 was excited at 488 nm, and the fluorescence emission at 525 nm was assessed with flow cytometry. (E) Western blot analysis for PARP upon the 48-hour treatment of AML cells with EAPB0203 and EAPB0503. (F) Proliferation assay after the treatment of AML cells (THP-1, MOLM-13, and OCI-AML3) with 1 μM EAPB0503 alone or in combination with 50 μM zVAD (general caspase inhibitor) for 24, 48, and 72 hours. Cell growth is represented as the percentage of the control as indicated. (G) Western blot analysis for p53, P-p53, p21, and GAPDH in OCI-AML3 and MOLM-13 cells treated for 48 hours as described. In all flow cytometry assays, histograms represent 1 of 3 independent experiments. *P* values less than .05 were considered significant (* $P \leq .05$, ** $P \leq .01$, *** $P \leq .001$). AML indicates acute myeloid leukemia; EAPB0203, 1-(2-phenylethyl)-*N*-methylimidazo[1,2-*a*]quinoxalin-4-amine; EAPB0503, 1-(3-methoxyphenyl)-*N*-methylimidazo[1,2-*a*]quinoxalin-4-amine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MMP, mitochondrial membrane potential; PARP, poly(adenosine diphosphate ribose) polymerase; PI, propidium iodide; P-p53, phosphorylated p53; zVAD: z-Val-Ala-DL-Asp(Ome)-fluoromethylketone Rhodamine 123 phosphorylated-p53.

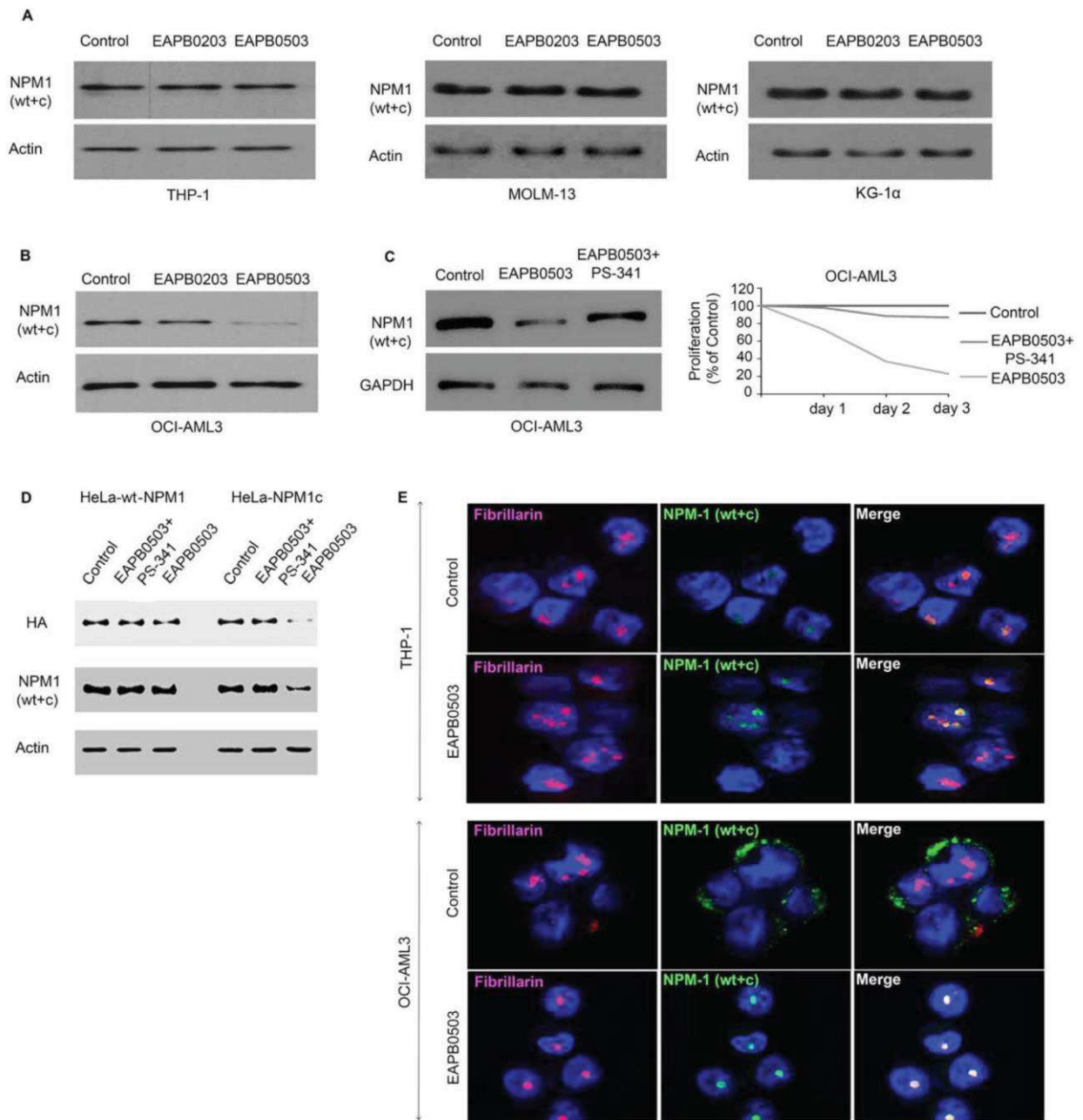


Figure 3. EAPB0503 induces proteasomal degradation of the NPM1c protein and restores the correct wt-NPM1 nucleolar localization in the *NPM1c* OCI-AML3 cell line. Western blot analysis of NPM1 recognizing both NPM1 (wt+c) and actin in (A) AML cell lines with *wt-NPM1* (THP-1, MOLM-13, and KG-1 α) and (B) *NPM1c* OCI-AML3 cell lines treated with 1 μ M EAPB0203 or EAPB0503 for 48 hours as indicated. (C) NPM1 (wt+c) and GAPDH in OCI-AML3 treated with 1 μ M EAPB0503 alone or in combination with 10 nM PS-341 (proteasome inhibitor) for 48 hours as indicated and proliferation assay after the treatment of OCI-AML3 with 1 μ M EAPB0503 alone or in combination with 10 nM PS-341 for 24, 48, and 72 hours. Cell growth is presented as the percentage of the control as indicated. (D) Western blot analysis for HA, NPM1 (wt+c), and actin in HeLa cells transfected with HA-tagged *wt-NPM1* or *NPM1c* and treated with 1 μ M EAPB0503 alone or in combination with 10 nM PS-341 for 48 hours as indicated. (E) Confocal microscopy analysis of NPM1 localization in THP-1 or OCI-AML3 cells after treatment with EAPB0503 for 48 hours. NPM1 was stained with an antibody recognizing NPM1 (wt+c) (green), nucleoli were stained with anti-fibrillarin (red), and nuclei were stained with 4',6-diamidino-2-phenylindole (blue). Images represent z-sections. AML indicates acute myeloid leukemia; EAPB0203, 1-(2-phenylethyl)-*N*-methylimidazo[1,2-*a*]quinoxalin-4-amine; EAPB0503, 1-(3-methoxyphenyl)-*N*-methylimidazo[1,2-*a*]quinoxalin-4-amine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HA, hemagglutinin; NPM1, nucleophosmin 1; wt-NPM1, wild-type nucleophosmin 1; NPM1c, cytoplasmic NPM1; NPM-1 (wt+c): wild type and cytoplasmic NPM1.

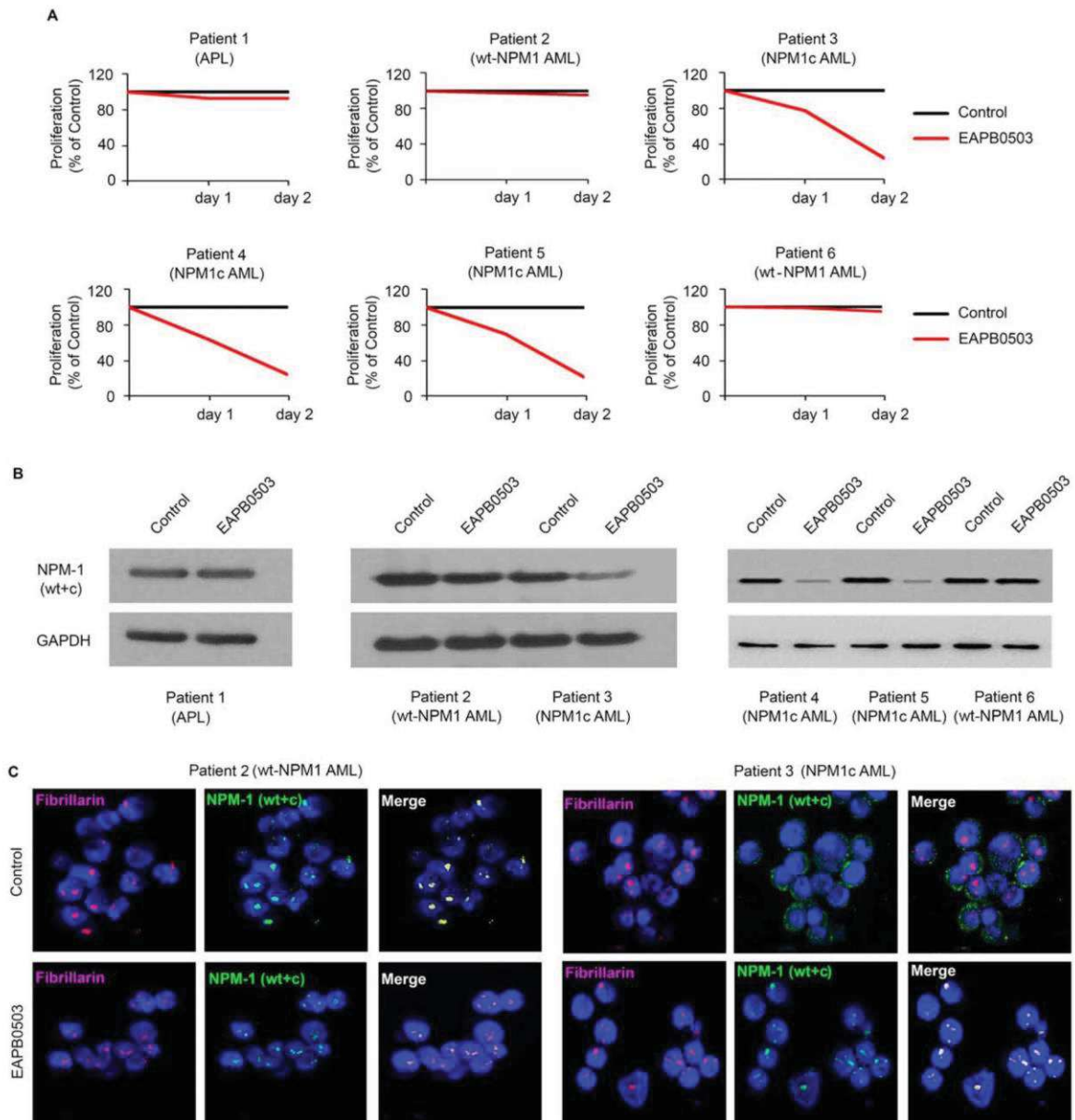


Figure 4. EAPB0503 inhibits proliferation, induces the degradation of NPM1c, and restores the nucleolar localization of *wt-NPM1* selectively in ex vivo treated blasts derived from *NPM1c* AML patients. Primary leukemic blasts were harvested from 3 patients and treated with 1 μ M EAPB0503. Patient 1 had APL with PML/RARA rearrangement, patients 2 and 6 were AML patients with *wt-NPM1*, and patients 3 to 5 were AML patients harboring an *NPM1* mutation without *FLT-3* internal tandem duplication. (A) Proliferation of AML blasts after treatment for 24, 48, and 72 hours. Cell growth is represented as the percentage of the control. (B) Western blot analysis for NPM1 (wt+c) and GAPDH in treated AML blasts as indicated previously. (C) Confocal microscopy of derived blasts from patients 2 and 3. NPM1 (wt+c) was stained with an anti-NPM1 (wt+c) antibody (green), nucleoli were stained with anti-fibrillarin (red), and nuclei were stained with 4',6-diamidino-2-phenylindole (blue). Images represent z-sections. AML indicates acute myeloid leukemia; APL, acute promyelocytic leukemia; EAPB0503, 1-(3-methoxyphenyl)-*N*-methylimidazo[1,2-*a*]quinoxalin-4-amine; FLT-3, FMS-like tyrosine kinase 3; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NPM1, nucleophosmin 1; wt-NPM1, wild-type nucleophosmin 1; NPM1c, cytoplasmic NPM1; NPM1 (wt+c), wild-type and cytoplasmic NPM1.

reduces the *NPM1c* AML BM burden in xenograft animals and warrants more preclinical investigation and then a clinical investigation.

DISCUSSION

In this report, we examine the effects of EAPB0503 and EAPB0203, 2 imidazoquinoxaline agents, on AML cell

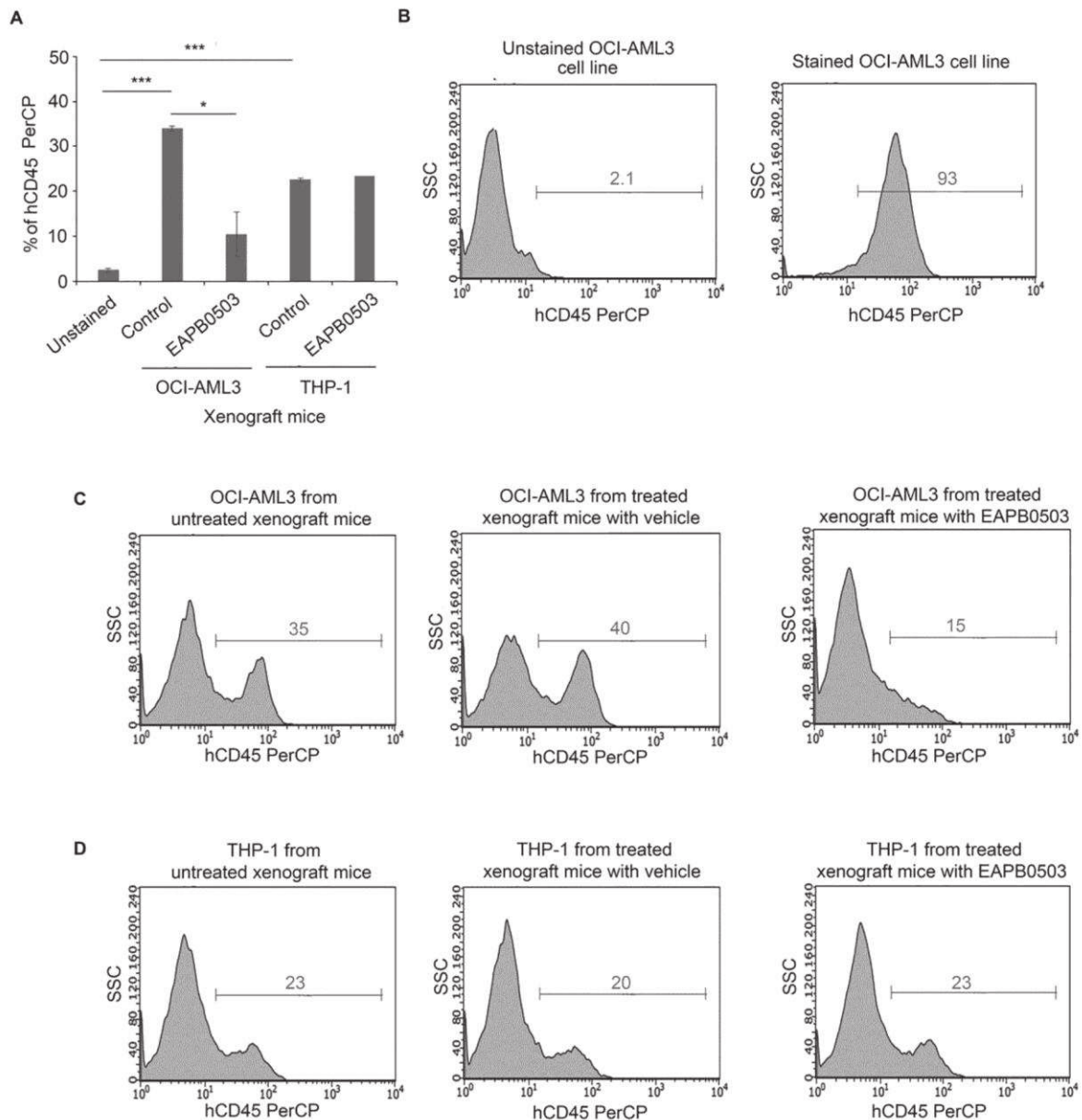


Figure 5. EAPB0503 selectively reduces the leukemia bone marrow burden in OCI-AML3 xenograft NSG mice. Eight-week-old female NSG mice were injected with 1×10^6 OCI-AML3 or THP-1 cells intravenously. EAPB0503 or its vehicle was administered for 5 days per week over a period of 2 weeks intraperitoneally. At the end of week 3, bone marrow was harvested from femurs and tibias of xenograft mice and then stained with the anti-hCD45 antibody. (A) Histograms showing the hCD45 PerCP percentage in xenograft animals. (B) Unstained and stained OCI-AML3 cell lines with the hCD45 antibody. (C) Representative histograms of stained and untreated OCI-AML3 xenograft mice, OCI-AML3 xenograft mice treated with the vehicle, and OCI-AML3 xenograft mice treated with EAPB0503. (D) Representative histograms of stained and untreated THP-1 xenograft mice, THP-1 xenograft mice treated with the vehicle, and THP-1 xenograft mice treated with EAPB0503. EAPB0503 indicates 1-(3-methoxyphenyl)-N-methylimidazo[1,2-a]quinoxalin-4-amine; NSG, NOD/Shi-*scid* *IL2r γ* ^{-/-}; PerCP: peridinin chlorophyll protein (**P* < .05, ***P* < .01, ****P* < .001); SSC, side scatter.

lines. Imidazoquinoxalines have arisen as promising anti-cancer drugs on the basis of their *in vitro* activity in T-cell leukemia and chronic myeloid leukemia and their *in vivo* activity in melanoma.^{22,23,26} We show that EAPB0503

has a specific growth-inhibition effect on *NPM1c* OCI-AML3 and IMS-M2 cells in a dose- and time-dependent manner. EAPB0503 activity in OCI-AML3 cells is considerably more pronounced than EAPB0203

activity, and this in line with its higher antitumor potency in other cancer types.^{24,25} Introducing *NPM1c* into cells harboring *wt-NPM1* sensitizes them to EAPB0503. The phenyl group is directly linked to the core imidazoquinoline heterocycle in EAPB0503, whereas an ethyl link exists in EAPB0203 between the 2 parts. This ethyl linker in EAPB0203 appears to abolish the antileukemic activity in most of the tested leukemia models in comparison with the direct linkage in the EAPB0503 compound.²⁶ Indeed, this change in the EAPB0503 structure enhanced its in vitro activity and led to better bioavailability in rats.²⁹

We have shown that EAPB0503 induces growth arrest and apoptosis in *NPM1c* AML cells. Apoptosis is accompanied by the dissipation of MMP and PARP cleavage, and this strongly suggests the involvement of the intrinsic apoptotic pathway. Our results are consistent with previous studies showing antitumor activity of EAPB0503 in melanoma and chronic myeloid leukemia with a mode of action similar to the mode of this compound.^{23,26}

NPM1c characterizes one-third of AML patients,^{6,10} and when it alone is present in the case of a normal karyotype, it confers a better prognosis.³⁵ *NPM1* mutations mediate malignancies as observed in transgenic and knock-in mice.³⁶ Mutated *NPM1* is the key hallmark of OCI-AML3 and IMS-M2 cells for maintaining their malignant proliferation. In *NPM1c* AML, emerging studies have shown that therapies targeting NPM1c oncoprotein degradation lead to inhibition of proliferation and the cell death of leukemic cells.¹⁴⁻¹⁶ In line with these findings, we have demonstrated that EAPB0503 degrades the NPM1c oncoprotein in a proteasome-dependent manner. This results in correcting the *wt-NPM1* nucleolar localization in both *NPM1c* AML cells and ex vivo treated blasts derived from *NPM1c* AML patients. Furthermore, in in vivo *NPM1c* AML xenograft animals, EAPB0503 showed a selective reduction of the BM leukemia burden.

Recently, EAPB0503 was shown to exert potent inhibition of tubulin polymerization that correlated with its antiproliferative activity.²⁷ Therefore, the corrective effect of *wt-NPM1* nucleolar localization after NPM1c degradation warrants testing the disruption of the microtubule network in *NPM1c* AML cells to further explain the mechanism of cell death.

Nowadays, most AML patients are still dying, especially because the basic therapies have remained unchanged or have only slightly changed over the last 2 decades. Nonetheless, before novel clinical therapies are introduced, a deep understanding of the therapeutic approach is required. The evolutionary changes emerging in

AML classification based on the morphology and cytogenetic/genetic changes reflect the importance of identifying the subtype-specific biology to determine the appropriate targeted therapy triggering degradation of the byproducts of these genetic modifications.¹³ Our results suggest that EAPB0503 holds promise for the treatment of *NPM1c* AML, especially in those patients with mutation A,³⁷ which represents 80% of *NPM1* mutations in AML³⁸ and is the hallmark mutation present in OCI-AML3 and IMS-M2.³⁹ These promising results were translated in vivo: among treated mice, EAPB0503 decreased the BM leukemia burden only in *NPM1c* xenograft mice. Further in vivo studies (survival and organ infiltration) and ex vivo studies (treated blasts) are required for us to have a complete idea of EAPB0503's mechanism of action.

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CONFLICT OF INTEREST DISCLOSURES

The authors made no disclosures.

AUTHOR CONTRIBUTIONS

Ali I. Nabbouh: Performance of experiments and reporting to Hiba A. El Hajj. **Rita S. Hleihel:** Performance of experiments and reporting to Hiba A. El Hajj. **Jessica L. Saliba:** Performance of experiments and reporting to Hiba A. El Hajj. **Martin M. Karam:** Performance of experiments and reporting to Hiba A. El Hajj. **Maguy H. Hamie:** Performance of experiments and reporting to Hiba A. El Hajj. **Hsin-Chieh J.M. Wu:** Performance of experiments and reporting to Hiba A. El Hajj. **Caroline P. Berthier:** Performance of experiments and reporting to Hiba A. El Hajj. **Nadim M. Tawil:** Performance of experiments and reporting to Hiba A. El Hajj. **Pierre-Antoine A. Bonnet:** Planning of study. **Carine Deleuze-Masquefa:** Planning of study. **Hiba A. El Hajj:** Planning of study and writing of manuscript.

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6. Discussion

6.1. Conclusion

In this study, we examined the effect of EAPB0503 and EAPB0203, two imidazoquinoxaline agents on AML cell lines. Imidazoquinoxalines have arisen as promising anticancer drugs based on their *in vitro* activities on T-cell leukemia and chronic myeloid leukemia and *in vivo* activities on melanoma (Moarbess, El-Hajj et al. 2008, Deleuze-Masquefa, Moarbess et al. 2009). We show that EAPB0503 has a specific growth inhibition effect on the *NPM-1* mutant OCI-AML3 cell line in a dose- and time-dependent manner. EAPB0503 activity on OCI-AML3 cells is considerably more pronounced than that of the older compound EAPB0203, in line with the anti-tumor higher potency of EAPB0503 when compared to EAPB0203 or the mother compound Imiquimod in other cancer types (Moarbess, Deleuze-Masquefa et al. 2008, Khier, Deleuze-Masquéfa et al. 2010). Introducing *NPM-1c* into cells harboring wt-*NPM-1* sensitizes them to EAPB0503. The phenyl group is directly linked to the core imidazo- quinoxaline heterocycle in EAPB0503 whereas an ethyl link exists in EAPB0203 between the two parts. This ethyl linker in EAPB0203 appears to abolish to anti-leukemic activity, in most of the tested leukemia models, in comparison to the direct linkage in the EAPB0503 compound (Saliba, Deleuze-Masquéfa et al. 2014). Indeed, this change in EAPB0503 structure enhanced its *in vitro* activity and led to a better bioavailability in rats (Khier, Gattacceca et al. 2010).

We showed that EAPB0503 induced growth arrest and apoptosis in *NPM-1* mutant AML cells. Apoptosis was accompanied by the dissipation of MMP and PARP cleavage, strongly suggesting the involvement of the intrinsic apoptotic pathway. Our results are consistent with other previous studies showing the anti-tumor activity of EAPB0503 on melanoma, and chronic myeloid leukemia with a similar mode of action of this compound (Moarbess, El-Hajj et al. 2008), (Saliba, Deleuze-Masquéfa et al. 2014).

NPM-1 mutation characterizes one third of AML patients (Falini et al., 2009; Falini et al., 2005), and when present alone in case of normal karyotype, it confers a better prognosis (Schlenk, Döhner et al. 2009). *NPM-1* mutations mediate malignancy as observed in transgenic or knock-in mice (Cheng, Sportoletti et al. 2010). Mutated *NPM-1* is the key hallmark of OCI-AML3 or IMS-M2 cells to maintain their malignant proliferation. In *NPM-1c* AML, emerging studies showed that therapies targeting the NPM-1c oncoprotein degradation lead to inhibition of proliferation and cell death of leukemic cells (Balusu, Fiskus et al. 2011, El Hajj, Dassouki et al. 2015, Martelli, Gionfriddo et al. 2015). In line with these findings, we demonstrate that EAPB0503 degrades NPM-1c oncoprotein, in a proteasome-dependent manner. This results in correcting the wt-NPM-1 nucleolar localization in both *NPM-1c* AML cells and *ex-vivo* treated blasts derived from *NPM-1c* AML patients. Furthermore, *in vivo* *NPM-1c* AML xenograft animals, EAPB0503 showed a selective reduction of BM leukemia burden.

Recently, EAPB0503 was shown to exert a potent inhibition of the tubulin polymerization correlating with its antiproliferative activities (Zghaib, Guichou et al. 2016). Therefore, the corrective effect of the nucleolar localization of NPM-1wt upon the selective EAPB0503-induced degradation of the mutated NPM-1 warrants testing the disruption of the microtubule network in OCI-AML3 and *ex vivo* blasts derived from *NPM-1* mutant AML blasts, to further explain the mechanism of cell death.

Until nowadays, most AML patients are still dying, specially that the adopted basic therapies remained unchanged or adopted slight changes over the last two decades. Nonetheless, before introducing novel clinical therapies, a deep understanding of the therapeutic approach is required. The evolutionary changes emerging on AML classification according to the morphology and cytogenetic/genetic changes reflects the importance of identifying the subtype-specific biology to determine the appropriate targeted therapy triggering degradation of the byproducts of these genetic modifications (Burnett, Wetzler et

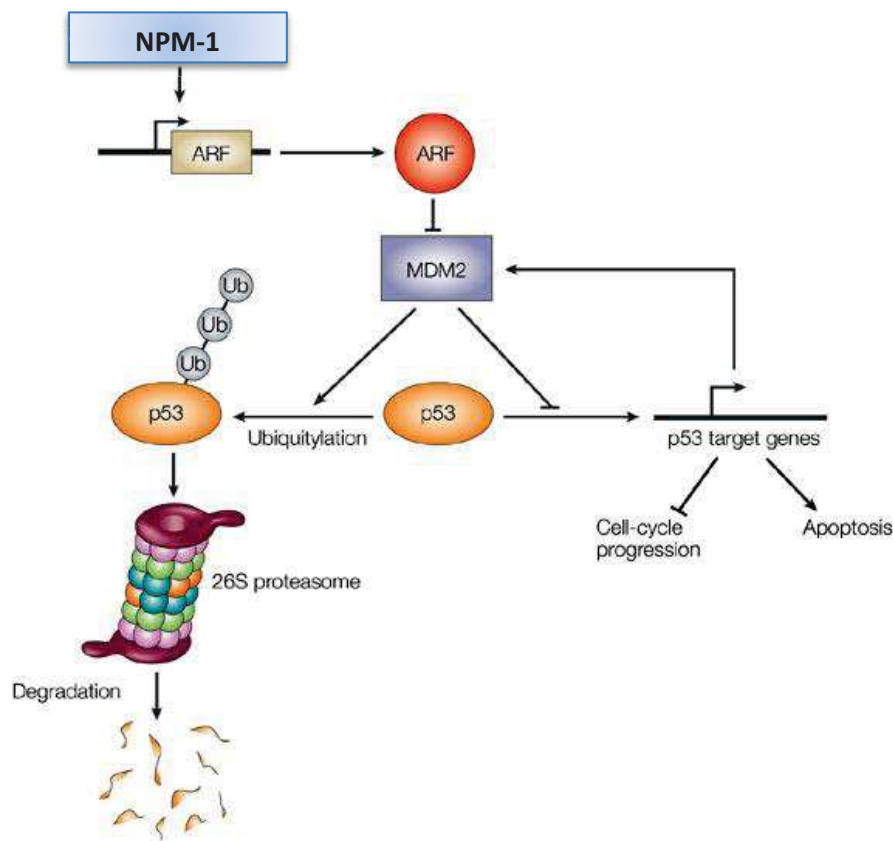
al. 2011). Our results suggest that EAPB0503 holds promising expectations in the treatment of *NPM-1c* AML, especially in those patients having mutation A (Sakamoto, Grant et al. 2015), which represents 80% of *NPM-1* mutations in AML (Wertheim and Bagg 2008), and the hallmark mutation present in OCI-AML3 and IMS-M2 (De Cola, Pietrangelo et al. 2014). These promising results were translated *in vivo* whereby only in *NPM1c* xenograft mice, EAPB0503 decreased BM leukemia burden in treated mice.

6.2. Perspectives

Further *in vitro* or *ex-vivo* studies on blasts from *NPM-1* mutated AML patients are required to have a clear idea about the mechanism of action of EAPB0503. Therefore, more patients' leukemic blasts would help confirming the activity of the drug. Furthermore, *in vivo* (survival and organ infiltration) on NPM-1 transgenic models (Cheng, Sportoletti et al. 2010) or xenografted mouse models (Falini, Martelli et al. 2008) using THP-1 and OCI AML-3 in NOG SCID mice are required to have a complete idea about the EAPB0503 mechanism of action.

Moreover, NPM-1 is known to interact with and enhance the stability of p53, upregulating its tumor suppressor function (Colombo, Marine et al. 2002) and preventing its proteasomal degradation by interacting with ARF and by ensuring it escapes its destruction through proteasomes (Kuo, den Besten et al. 2004, Colombo, Bonetti et al. 2005). NPM-1 is also known to interact with p53 by binding to and inactivating MDM2 (Grisendi, Mecucci et al. 2006) (Figure 38). Furthermore, NPM-1 is known to play an important role in ribosomal biogenesis (Falini, Martelli et al. 2008) and is known to oligomerize with the NPM-1c where it will continuously translocate to the cytoplasm.

Figure 38. The ARF–MDM2–p53 pathway



The increase in ARF transcription and its binding to MDM2 inhibits the degradation of p53. Thus, the increase of p53 activity leads and cell-cycle arrest or apoptosis (Hipfner and Cohen 2004).

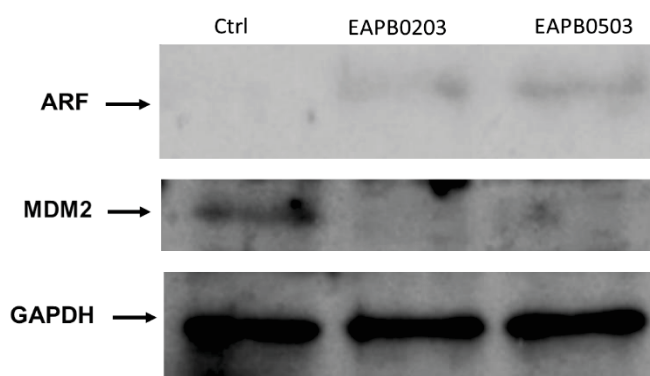
Ub: ubiquitin.

Assessing the ribosome biogenesis that controls cell proliferation (Thomas 2000) by probing the whole “ribosomal protein-Mdm2-p53 pathway” that includes ribosomal proteins (RPs), ARF, MDM2 and p53 will help providing a clearer idea about the mode of action of EAPB0503 that we have shown in the current study to degrade the NPM-1c oncoprotein.

In order to investigate how our drugs, particularly EAPB0503, affect the interaction of NPM-1 with p53 and its tumor suppressor function, we started to determine the link between the degradation of the NPM-1c and the P53 pathway by assessing the modulation of ARF and MDM2 at the protein expression level.

Our preliminary results show that the stabilization of p53 is maintained *via* MDM2 degradation, and is promoted by ARF upregulation at 48 hours' post-treatment (Figure 39).

Figure 39. Western blot analysis for ARF and MDM2 in OCI-AML3 cells.



EAPB0503 upregulates ARF that promotes MDM2 degradation and stabilizes p53. Cells were treated with 1 μ M of EAPB0203 and EAPB0503 for 48 hours. Whole SDS protein lysates from untreated and treated AML cells probed with specific antibodies for *NPM-1* or GAPDH.

The results are very preliminary and should investigate the MDM2 inactivation/degradation to elaborate more on the mode of action of EAPB0503. Moreover, ribosomal proteins (Rps) expression (Federici and Falini 2013) will be investigated to give a clearer idea on how our drug affects the whole “ribosomal protein-Mdm2-p53 pathway” through NPM-1c degradation.

Last but not least, microtubules are very essential components in preserving the cytoskeleton of cells and are involved in many functions such as mitosis, maintenance of cellular morphology and other major functions (Janke and Kneussel 2010, Ma, Fang et al. 2013). Due to their in cell division, microtubule dynamics is considered as a good target in cancer treatment (Jordan, Hadfield et al. 1998, Giannakakou, Sackett et al. 2000, Kamal, Reddy et al. 2014). In a very recent study, EAPB0503 revealed highly significant inhibition of the tubulin polymerization (up to 84 %) resulted in significant anti-proliferative activity against human melanoma cells (Zghaib, Guichou et al. 2016). This will encourage us to assess the effect of EAPB0503 on the inhibition of the tubulin polymerization in AML cells by testing its binding ability to tubulin.

Furthermore, the proteasomal mediated NPM-1c degradation requires an investigation of the ubiquitin-proteasome pathway, and the effect of EAPB0503 on the NPM-1c SUMoylation and ubiquitylation before its degradation to check whether the post-translational modifications induced by the treatment can be similar to those described by Dassouki et al. 2015 on Tax oncoprotein in ATL.

Finally, since Nuclear Bodies (NBs) are involved in pathogenesis and response to therapy in acute promyelocytic leukemia (APL) (Ablain, Rice et al. 2014),

In APL, it was demonstrated that PML/RARA degradation restores PML NBs and activates a p53 that leads to APL eradication (Ablain, Leiva et al. 2013, dos Santos, Kats et al. 2013, Vitaliano-Prunier, Halftermeyer et al. 2014).

In *NPM-1* mutant AML, a recent study by our laboratory suggested a role of PML and/or SUMO NBs in the degradation process of NPM-1c, the results obtained with *NPM-1* mutant AML interestingly revealed some similarities as in APL upon treatment with RA/arsenic. Mutant NPM-1 was degraded, p53 signaling was activated and PML NBs nuclear organization were restored ex vivo and in vivo (El Hajj, Dassouki et al. 2015). It will be interesting to investigate the effect of EAPB0503 on the restoration or the enhancement of PML/SUMO NBs in AML cells that harbors the *NPM-1* mutation as the restoration of NBs that could contribute to the therapeutic efficacy as for ATRA/Arsenic in APL (Ablain, Rice et al. 2014, Lehmann-Che, Bally et al. 2014). Moreover, and since SUMOylation is an important NPM1c post-translational modification leading to its degradation, an eye glance will be focusing on the SUMO-deconjugating proteases (sentrin-specific protease or SENP) (Lindström 2010) involved in removing the SUMO-tag from a protein target, in the context of NPM1c AML. Among the 7 described SENPs, SENP3 and SENP5 are known to be localized within the nucleoli and bind NPM-1 (Yun, Wang et al. 2008). SENP3 and SENP5 expression versus localization should be investigated in the context of NPM-1c AML and thereafter following treatment with EAPB0305. Since SENP3 can remove SUMO from

NPM-1 (Gonda, Wudel et al. 2006, Nishida and Yamada 2008) and its turnover is promoted by ARF (Kuo, den Besten et al. 2008), a look at the SENP3 protein expression levels would help us having a clearer idea about the interactions between NPM1, ubiquitin and SUMO pathways that play important roles in maintaining nucleolar structure and function.

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Résumé

La leucémie myéloïde aiguë (LMA) est une maladie clonale hétérogène caractérisée par une prolifération immature des cellules myéloïdes et une défaillance de la moelle osseuse. Malgré les avancées rapides dans le domaine de la LMA, notamment concernant de nouvelles cibles thérapeutiques et une meilleure compréhension des mécanismes biologiques, le traitement clinique de la LMA reste inchangé et dépend du caryotype des patients. Lors des trois dernières décennies, la plupart des patients ont fini par récidiver et décéder de la maladie ; il n'y a encore aucun schéma thérapeutique standard qui améliore le pronostic et le traitement de la LMA.

Les imidazoquinoxalines sont des dérivés de l'imiquimod qui présentent un effet immunomodulateur indirect et une activité anti-tumorale directe contre le mélanome et le lymphome à cellules T, provoquant l'inhibition de la croissance cellulaire et l'induction de l'apoptose par la voie dépendante des caspases. Nous avons étudié les effets des dérivés de la série imidazoquinoxaline, EAPB0203 et EAPB0503, sur des lignées de cellules humaines LMA. Nous avons montré que EAPB0503 inhibe la croissance de la lignée cellulaire LMA qui présente la mutation *NPM-1* de manière dose et temps dépendants. Par rapport au dérivé EAPB0203 précédemment synthétisé, EAPB0503 a une activité inhibitrice plus forte sur les cellules OCI-AML3 ainsi que sur des cellules provenant de patients LMA. Nous avons démontré que EAPB0503 induit une dégradation médiée par les protéasomes de NPM-1 muté ainsi qu'un rétablissement de la localisation nucléolaire de NPM-1 sauvage conduisant à une inhibition de la prolifération des cellules OCI-AML3.

EAPB0503 induit une apoptose massive comme démontré par l'analyse du cycle cellulaire avec une accumulation de cellules traitées en phase preG₀. L'apoptose a été confirmée par la réponse positive au test de l'annexine V, le clivage de PARP et la dissipation du potentiel membranaire mitochondrial dans les cellules OCI-AML3 traitées.

En outre, EAPB0503 a augmenté les niveaux d'expression et de phosphorylation de p53.

Ces résultats, concernant l'arrêt de la croissance cellulaire et l'apoptose, sélectivement dans les cellules LMA présentant la mutation *NPM-1*, renforcent l'idée d'un ciblage de l'oncoprotéine NPM-1 muté pour éliminer les cellules leucémiques et justifient une évaluation préclinique plus large puis une évaluation clinique pour ce candidat médicament prometteur.

En conclusion, nos études mettent en évidence l'utilisation d'EAPB0503 comme un candidat médicament prometteur qui présente une activité anti-tumorale encourageante et qui devrait faire l'objet d'études précliniques dans le cadre d'une thérapie ciblée contre la LMA.

Mots clés : Leucémies, Lymphomes, Imidazoquinoxalines, Analyses in vitro, Modèles murins

Abstract

Acute myeloid leukemia (AML) is a heterogeneous clonal disorder characterized by immature myeloid cell proliferation and bone marrow failure. Although the remarkable improvements in the field and regarding new drug targets and better understanding of the biology, the clinical treatment of AML remains unchanged and depending on karyotype of patients. For the last thirty years with the majority of patients, in the end, relapsing and dying of the disease, there is no standard regimen that improves prognosis and treat AML yet.

Imidazoquinoxalines are imiquimod derivatives with indirect immunomodulatory effect and direct antitumor activity on melanoma and T-cell lymphoma, attributed to growth inhibition and induction of apoptosis through caspase-dependent pathway. We examined the effects of imidazoquinoxaline derivatives, EAPB0203 and EAPB0503, on human AML cells. We found that EAPB0503 inhibit cell growth of AML cell line that harbors the NPM-1 mutation in a time- and dose-dependent way. Compared to the previously synthesized EAPB0203, EAPB0503 has a more pronounced inhibitory activity on OCI-AML3 cells and cells derived from AML patients as well. We demonstrated that the EAPB0503 induces proteasome-mediated degradation of mutant NPM-1, and restoration of the nucleolar localization of the NPM-1wt leading to an inhibition of the proliferation of OCI-AML3 cells.

EAPB0503 induced massive apoptosis as demonstrated with the cell cycle analysis by the accumulation of treated cells in the preG₀ region. Apoptosis has been confirmed by Annexin V positivity, PARP cleavage, and dissipation of mitochondrial membrane potential in treated OCI-AML3 cells.

Furthermore, EAPB0503 increased the expression and phosphorylation levels of p53.

These results in growth inhibition and apoptosis, selectively in AML cells that harbor the NPM-1 mutation reinforce the idea targeting NPM-1m oncoprotein to eradicate leukemic cells and warrant a broader preclinical then clinical evaluation of this promising drug.

In conclusion, our studies highlight the use of EAPB0503 as a promising anti-tumor activity to be investigated preclinically in AML targeted therapy.

Keywords: Leukemia, Lymphoma, Imidazoquinoxalines, in vitro analyzes, murine models