

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

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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;123:1662-73. © 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

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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 pBye 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* *IL2 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

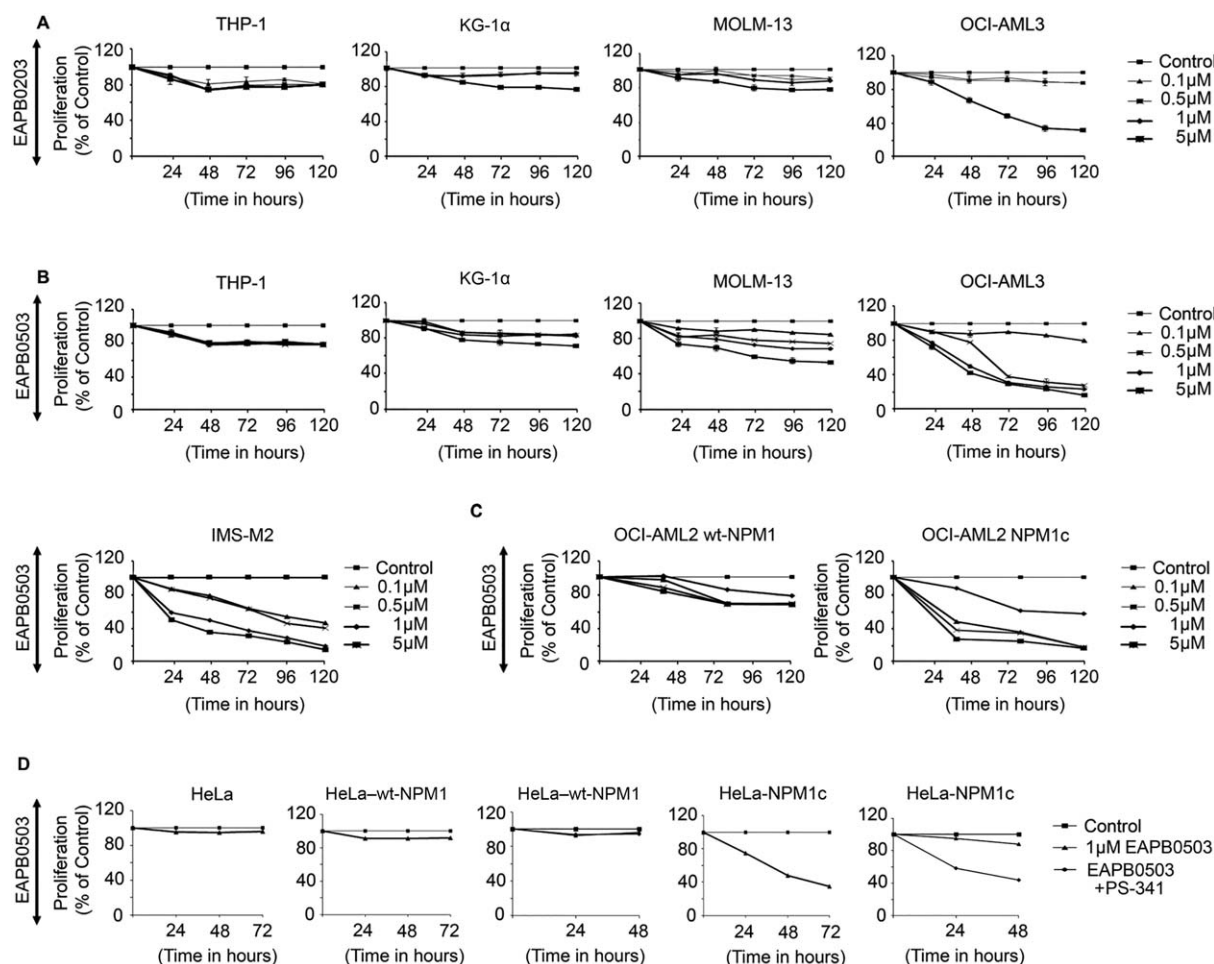


Figure 1. EAPB0503 induces selective growth inhibition in *NPM1c* AML cells. AML cell lines with normal *NPM1* (THP-1, KG-1α, and MOLM-13) and *NPM1c* (OCI-AML3 and IMS-M2) were treated with increasing concentrations (0.1–5 μM) of (A) EAPB0203 and (B) EAPB0503 for 24, 48, 72, 96, and 120 hours. (C) Stably 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 EAP0503 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

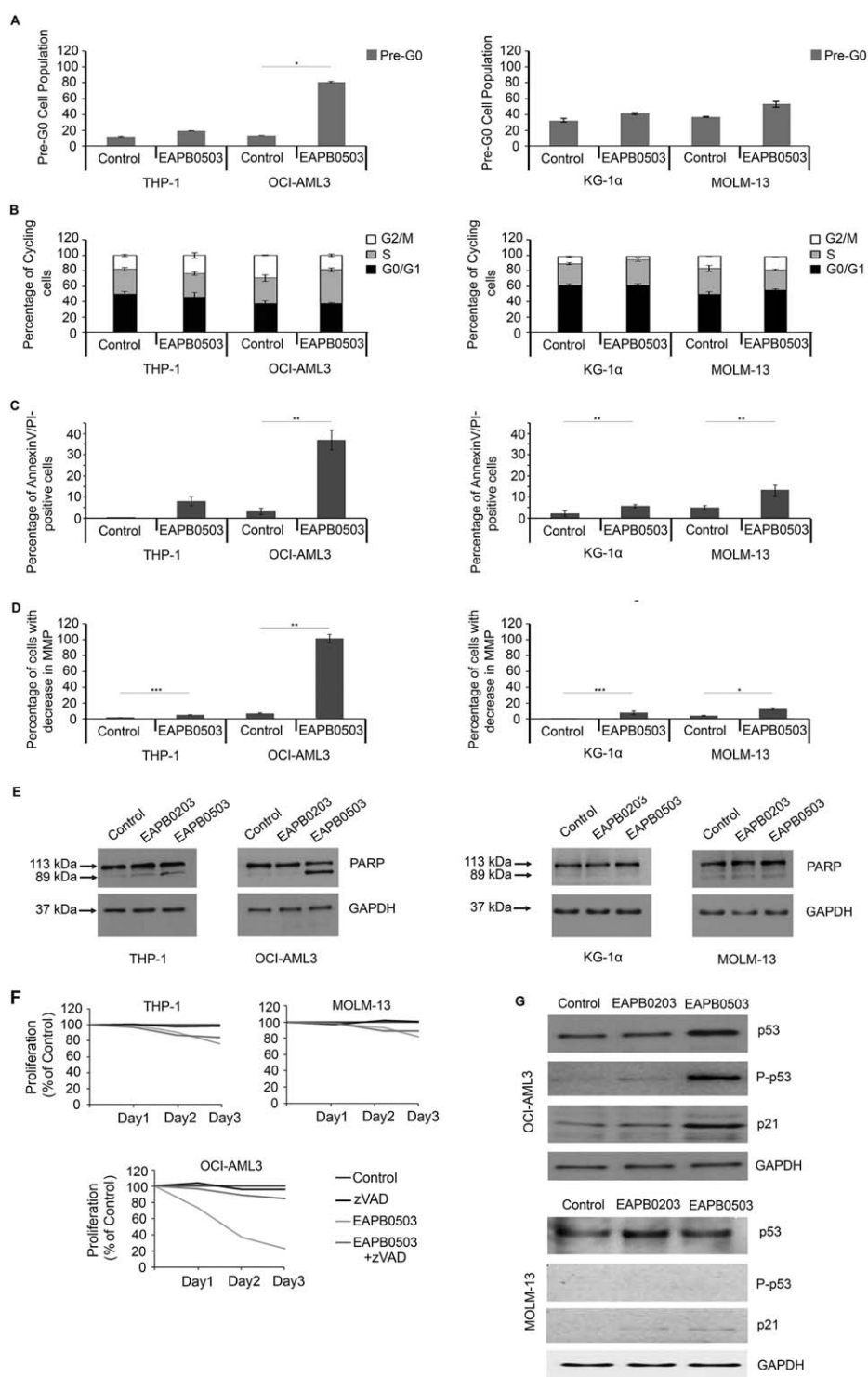


Figure 2.

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.

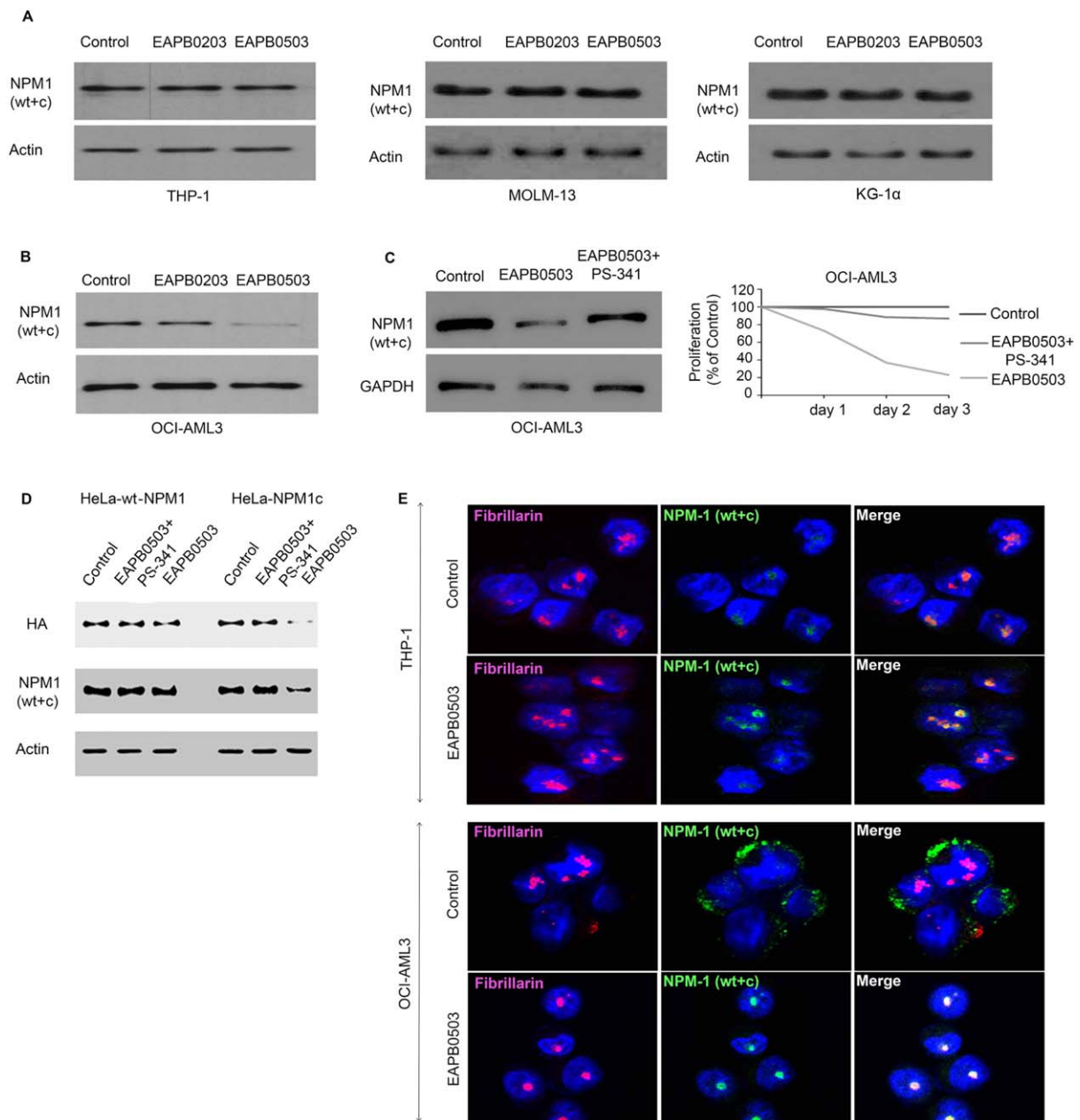


Figure 3. EAPB0503 induces proteasomal degradation of the NPM1c protein and restores the correct wt-NPM1 nucleolar localization in the *NPM1c* OCI-AML3 cell line. Western blot analysis of NPM1 recognizing both NPM1 (wt+c) and actin in (A) AML cell lines with *wt-NPM1* (THP-1, MOLM-13, and KG-1 α) and (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.

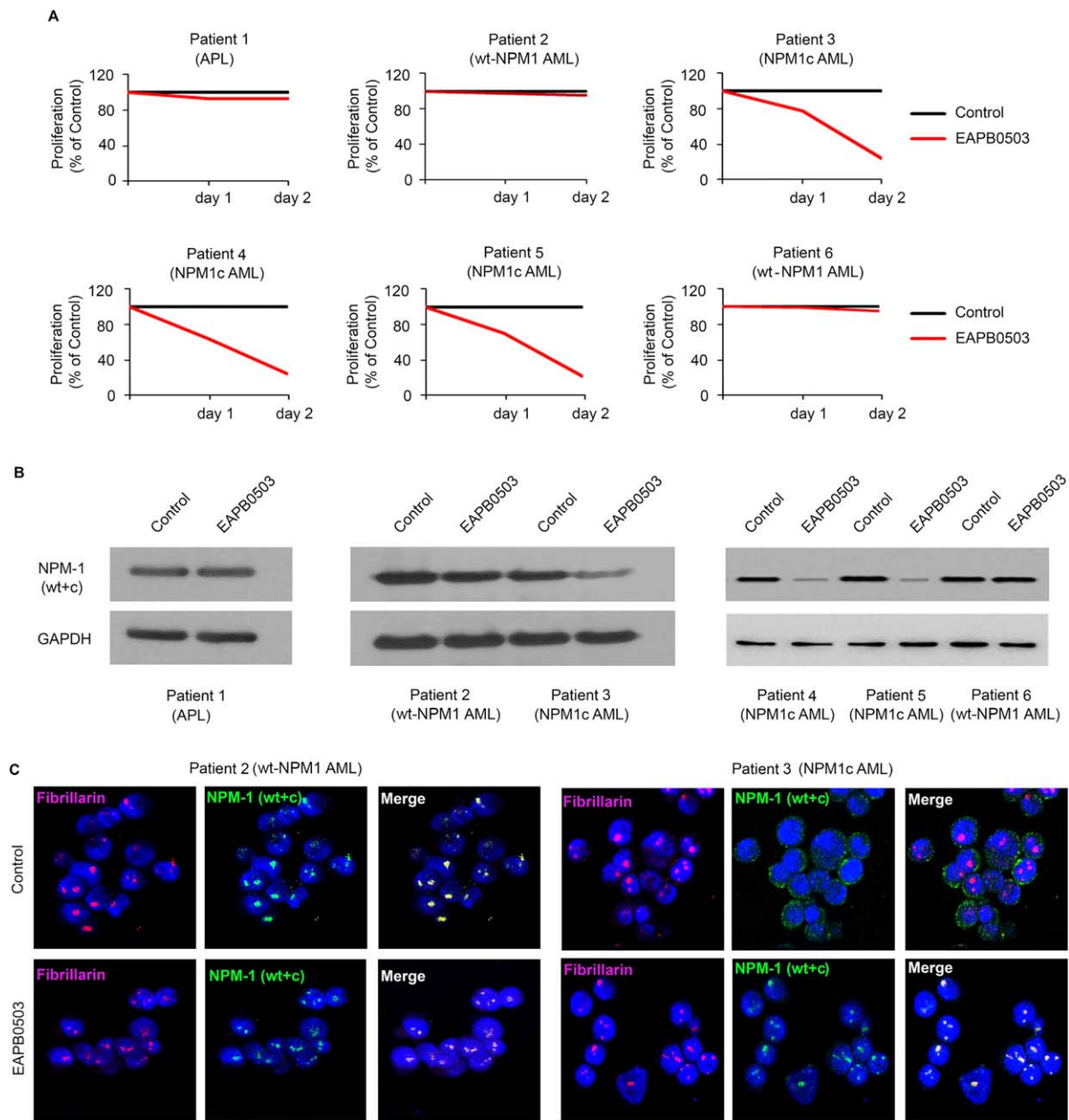


Figure 4. EAPB0503 inhibits proliferation, induces the degradation of NPM1c, and restores the nucleolar localization of *wt-NPM1* selectively in ex vivo treated blasts derived from *NPM1c* AML patients. Primary leukemic blasts were harvested from 3 patients and treated with 1 μ M EAPB0503. Patient 1 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

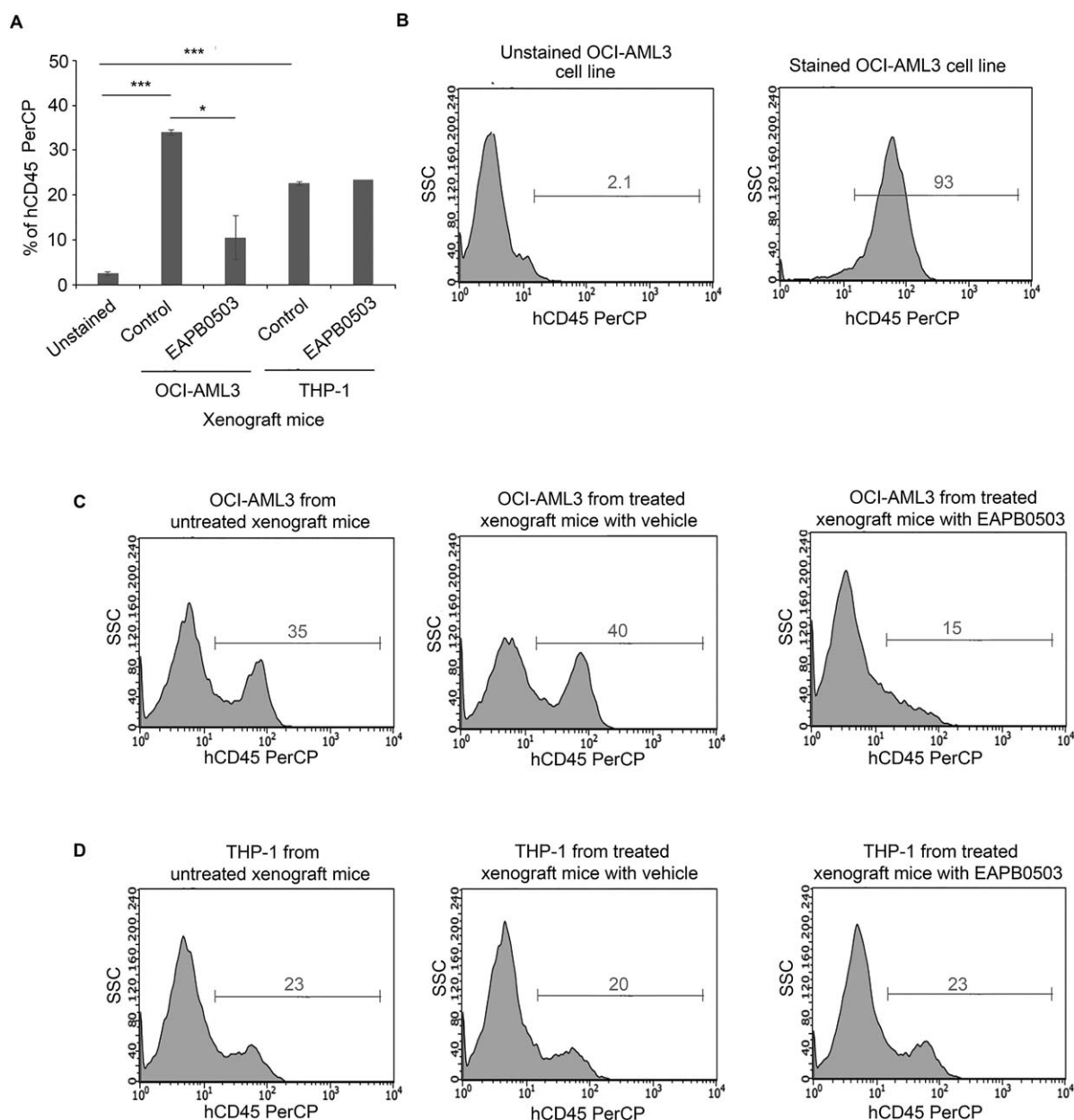


Figure 5. EAPB0503 selectively reduces the leukemia bone marrow burden in OCI-AML3 xenograft NSG mice. Eight-week-old female NSG mice were injected with 1×10^6 OCI-AML3 or THP-1 cells intravenously. EAPB0503 or its vehicle was administered for 5 days per week over a period of 2 weeks intraperitoneally. 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* *IL2 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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