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15-Methylene-Eburnamonine Kills Leukemic Stem Cells and Reduces Engraftment in a Humanized Bone Marrow Xenograft Mouse Model of Leukemia

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Abstract

Recent studies suggest that leukemia stem cells (LSCs) play a critical role in initiation, propagation, and relapse of leukemia. Herein we show that (–)-15-methylene-eburnamonine, a derivative of the alkaloid, (–)-eburnamonine, is cytotoxic against acute and chronic lymphocytic leukemias (ALL and CLL) and acute myelogenous leukemia (AML). The agent also reduces primary LSC frequency *in vitro*. The cytotoxic effects appear to be mediated via the oxidative stress pathways. Furthermore, we show that the compound kills AML, ALL, and CLL stem cells, and using a novel humanized bone marrow murine model of leukemia (huBM/NSG), it reduces progenitor cell engraftment.

Graphical abstract

A derivative of the alkaloid eburnamonine, (–)-15-methylene-eburnamonine, is cytotoxic against acute and chronic lymphocytic leukemias and acute myelogenous leukemia. The compound kills primary leukemia stem cells, and using a novel humanized bone marrow murine model of leukemia, it reduces progenitor cell engraftment.

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Keywords

leukemia; cancer stem cells; natural products; xenograft models; glutathione

Introduction

Leukemia refers to a spectrum of hematological malignancies including acute myelogenous leukemia (AML), acute lymphocytic leukemia (ALL), chronic myelogenous leukemia (CML), and chronic lymphocytic leukemia (CLL).¹ According to the National Cancer Institute's Surveillance, Epidemiology, and End Results (SEER) program, nearly 50,000 new cases of leukemia were diagnosed in 2013.² Although the use of novel therapies, such as tyrosine kinase inhibitors, have improved treatment outcomes, the majority of patients demonstrate a relentless progressive-relapsing course and may remain on treatment for years, ultimately succumbing to their disease.³ Relapse of leukemia is attributed to a subpopulation of cells defined as leukemia stem cells (LSCs) that are resistant to current therapeutics and are capable of disease re-initiation.⁴⁻⁷ Thus, to achieve complete remission of leukemia, the development of novel therapeutics that target LSCs is imperative. Even though LSC-directed therapeutics are emerging,⁸⁻¹⁰ detailed preclinical evaluation of these agents is limited by a lack of appropriate animal models. Preclinical animal models are useful tools to better understand tumor initiation and progression and to predict the activity of an anticancer agent in the clinic. Ideally, these models should recapitulate the biological characteristics of the tumor and of the related tumor microenvironment. The physiological environment in which LSCs reside has been shown to play a critical role in the maintenance of stem cell characteristics, such as pluripotency and self-renewal.^{11,12} Indeed, it was recently shown that bone marrow (BM) mesenchymal stem cells rescued CLL cells from spontaneously undergoing apoptosis following treatment with various drugs.¹³ A number of studies suggested that LSCs reside in the bone marrow microenvironment composed of the extracellular matrix (ECM) proteins, such as collagens I and IV, hyaluronic acid, fibronectin and laminin, and stromal components, such as mesenchymal stem cells, fibroblasts, osteoblasts, osteoclasts, and adipocytes.¹²⁻¹⁴ NOD.Cg-Prkdc^{scid} IL2rg^{tm1Wjl}/SzJ (NSG) mice have been shown to be superior recipients for xenotransplants, immunity reconstitution, and tumorigenicity studies compared to SCID or even NOD/SCID mice due to their severe immunodeficiency.¹⁵ Hence, we generated a humanized bone marrow murine model of leukemia, in which the mouse bone marrow was humanized by engrafting human bone marrow mesenchymal stem cells (BMSCs) into the tibias of NSG mice. Co-

transplantation of leukemia cells with BMSCs resulted in the colonization of bone by the primary leukemia cells leading to the establishment of clinical manifestations of disease.

Natural products have long been a source of inspiration for drug discovery, especially in the development of new anticancer agents.¹⁶ The Madagascar periwinkle (*Catharanthus roseus*), which produces the cytotoxic vinca alkaloids, vinblastine and vincristine, is one of the primary sources of naturally derived chemotherapies.¹⁷ The alkaloids, (–)-eburnamonine (**1**) and vincamine (**2**), are also isolated from the Madagascar periwinkle (Figure 1),¹⁸ and display a favorable pharmacokinetic profile.¹⁹ Recently, we designed and synthesized (–)-15-methylene-eburnamonine (**3**) to impart antiproliferative activity through structural modification.²⁰ Specifically, the analogue **3** displays a critical enone group,²¹ and this functional group imparts cytotoxic properties similar to other enone-bearing natural products, such as parthenolide (**4**)²² and andrographolide (**5**).²³ Additionally, **3** was shown to exert antiproliferative activity against human multiple myeloma, breast, and prostate cancer cell lines *in vitro*.²¹ Herein, we show that (–)-15-methylene-eburnamonine (**3**) is cytotoxic against primary ALL, AML, and CLL cells and primary LSCs *in vitro*. Cytotoxicity appears to be attenuated by glutathione, suggesting the potential involvement of oxidative stress pathways. Furthermore, using a huBM/NSG model, we show that agent **3** reduces AML, ALL, and CLL stem and progenitor cell engraftment in NSG mice. Taken together these results demonstrate the utility of a novel mouse model for evaluating the efficacy of anti-leukemia therapeutics in the context of the tumor microenvironment and highlight the potential of **3** as a clinical candidate for leukemia therapy with the potential to eliminate leukemia stem cells and progenitor cells.

Results and Discussion

A preliminary screen in HL-60 (human acute promyelocytic leukemia) cells with **1** and **3** identified antiproliferative effects.²⁰ Additional evaluation of **1** and **3** side-by-side in MDA-MB-231 (breast cancer),²⁰ LNCaP (prostate cancer), RPMI-8226 (myeloma), U266 (myeloma), and KMS-12BM (myeloma) cell lines²¹ is shown in Table 1. To investigate further the cytotoxicity of **3** in leukemia cells, primary ALL, AML, and CLL cells were treated with varying doses of **3** and cell viability was assessed by calcein AM staining (Figure 2). All types of primary leukemia cells were strongly impaired by increasing doses of **3** with mean viability of 55% for AML (n = 3 patients) and 75% for ALL (n = 3 patients) at 72 hours (30 μ M, **3**) (Figs. 2B and 2C, respectively). As even untreated primary CLL samples exhibited low viability at 72 hours, the effect of **3** on these samples was determined 48 hours post treatment. Treatment with 30 μ M of **3** resulted in a mean viability of 35% for CLL (n = 3 patients) at 48 hours (Figure 2D). Thus ALL, AML and CLL cells appear to be susceptible to the cytotoxic effects of **3**.

Development of therapeutics that target LSCs is imperative for achieving complete remission of disease. Thus, we next examined whether the cytotoxic effects of **3** were also directed against phenotypically described AML and ALL stem cells. Stem cell subpopulations were quantified by gating the CD34⁺CD38[–] population for AML²⁴ and CLL²⁵ and the CD34⁺CD10[–] population for ALL.⁶ Treatment with **3** resulted in reduction of CD34⁺CD38[–] cells in AML specimens and CD34⁺CD10[–] cells in ALL specimens

(Figure 3). Whereas treatment with 15, 30 and 50 μM of **3** for 72 hours resulted in ~80% reduction of $\text{CD34}^+\text{CD38}^-$ cells in AML specimens ($n = 3$ patients), the same concentrations reduced the $\text{CD34}^+\text{CD10}^-$ cells in ALL specimens ($n = 3$ patients) in a dose-dependent manner. Unfortunately, the low viability of bulk CLL cells hindered our ability to determine the effects of **3** on CLL LSCs; however, **3** does appear to reduce AML and ALL LSCs. Moreover, these data illustrate that **3** affects both differentiated and stem cell populations of the leukemia models.

Originally, **3** was designed by installing an exocyclic enone into the structure of **1** (see Figure 1).²⁰ Subsequent studies showed that this functional group is not only required for activity but also susceptible to conjugation by reactive thiols.²¹ In a similar fashion, this functional group imparts biological activity to another natural product, parthenolide (**4**), and this compound has been shown to eliminate LSCs by increasing intracellular reactive oxygen species (ROS)⁸ and depleting intracellular glutathione levels.²⁶ To examine if the anti-leukemia properties of **3** were also related to these processes, we co-treated cells with **3** and the antioxidant, glutathione (Figure 4). This resulted in the blockade of the cell death response in primary AML and ALL cells induced by **3**. Staining with calcein AM and ethidium bromide depicts that the effects of **3** were abrogated by the addition of glutathione (Figure 4A). These data provide some additional evidence that oxidative stress or glutathione depletion may mediate the anti-leukemic properties of **3**. The likely mechanism is addition of glutathione to the enone of **3**.²¹

Recent studies have utilized xenograft models of AML and ALL to characterize and assess therapeutics against these diseases. In contrast, efforts at generating xenograft models of CLL have been hindered by extremely inefficient engraftment of primary CLL cells in immunodeficient hosts.^{27,28} We hypothesized that providing a microenvironment using immortalized human bone marrow mesenchymal stem cell line (hTERT-MSK) would also assist engraftment of primary CLL cells. To test this hypothesis, NSG mice were co-transplanted with hTERT-MSKs and primary CLL cells ($n = 3$ patients) and were monitored for signs of disease.²⁹ In addition, we also co-transplanted primary ALL ($n = 3$ patients) and AML cells ($n = 3$ patients) with hTERT-MSKs to create xenograft models of these diseases that could be used to assess the effect of the bone marrow microenvironment on drug efficacy. Survival of mice receiving co-transplanted primary AML, ALL, and CLL cells was substantially low, as anticipated (Figure S1). Mice injected with AML cells developed symptoms of disease such as weakness and scruffiness around 150 days post transplantation and exhibited enlarged cervical lymph nodes (Figure S2).²⁹ Animals receiving ALL cells developed weakness, lethargy and bloating of the abdominal region 60 days post transplantation due to enlarged spleens (Figure S2). Mice receiving CLL cells were moribund by 150 days post transplantation (Figure S2). However, these mice did not exhibit enlarged lymphoid organs. Infiltration of cells expressing human HLA⁺ and CD45⁺ antigens were found bilaterally, in the bone marrow isolated from the left and right femurs and tibias as well as in the peripheral blood collected from these animals (Figure S3). This indicated successful BM engraftment and systemic spread of primary AML, ALL, and CLL in huBM/NSG model.

To evaluate the ability of **3** to kill LSCs, NSG mice were injected with leukemia cells 24 hours post treatment with 30 μ M of **3**. A shorter treatment time was chosen to ensure sufficient numbers of viable cells were available for injection. As LSCs have been shown to be responsible for leukemia cell engraftment in immunodeficient mice,¹ we hypothesized that reduction of LSCs by **3** will result in lower engraftment efficiency of leukemia cells in these animals. Mice were co-injected with primary leukemia cells pretreated with DMSO vehicle control or agent **3** and hTERT-MSCs to create a humanized bone marrow microenvironment. One hundred and fifty days post injection, mice receiving AML cells treated with the vehicle control began to experience weakness and weight loss and exhibited the signs of bone disease marked by hunched vertebra and systemic presence of leukemia presenting with enlarged spleens (Figure 5). In comparison, mice receiving AML cells treated with **3** did not display external signs of disease and exhibited smaller spleens compared to mice receiving DMSO vehicle control treated AML cells (Figure 5A). Similarly, mice receiving ALL cells treated with **3** did not develop enlarged cervical lymph nodes which were observed in mice receiving DMSO vehicle control treated ALL cells (Figure 5D). Pre-treatment with **3** also prevented weight loss in mice receiving CLL cells compared to mice receiving CLL cells pre-treated with DMSO vehicle control (Figure 5E). Thus, pretreatment of leukemia cells with **3** reduces leukemia disease severity in mice.

To confirm that the reduction in disease severity was the result of the reduction in the numbers of LSC after treatment with compound **3**, mice injected with leukemia cells treated for 24 hours with 30 μ M of **3** were euthanized 8 weeks post injection and examined for the presence of LSCs. Mice injected with **3** in all three types of leukemia exhibited lower numbers of phenotypically described LSCs in the bone marrow compared to mice injected with DMSO vehicle control (Figure 6). Taken together, this data suggests that **3** reduces the numbers of LSC, and thus, subsequent engraftment in mice thereby preventing disease severity.

Conclusions

The development and characterization of novel therapeutics that eliminate LSCs is imperative to achieve complete remission, and possibly a cure, for leukemia. In the present study, we present a novel xenograft model of leukemia with a humanized bone marrow microenvironment generated by injecting immortalized human bone marrow mesenchymal stem cells directly into the tibias of immunodeficient mice. Mesenchymal stem/stromal cells (MSCs) are stromal progenitors with multi-lineage differentiation potential toward mesodermal cell lineages. Numerous studies have established that MSCs provide important survival and drug resistance signals to leukemia cells.^{30–33} AML LSCs have been shown to attach to the bone marrow microenvironment via interactions between very late antigen-4 (VLA4) on leukemic cells and fibronectin on MSCs.³³ This interaction between MSCs and LSCs has been shown to be critical for survival and persistence of LSCs. Antibody mediated blockade of VLA-4, in conjunction with cytarabine, prevented the development of AML in a xenograft model, presumably by mobilization and induction of LSC differentiation, thereby sensitizing them to cytarabine mediated toxicity. In another study, CD146⁺ MSCs were shown to express CXCL12, which promotes LSC homing to the bone marrow niche. MSCs

have also been shown to protect CLL cells from spontaneous and drug-induced apoptosis.³² Cell-cell contact was required for protection of CLL cells from fludarabine induced apoptosis and the protective effect of MSCs was absent when CLL cells were separated from the MSCs by micropore filters. In addition, MSCs give rise to osteoblasts, which also play an important role in regulating LSC function.³¹ These observations underscore the effects of MSC-LSC cross talk on drug activity. In this light, the humanized bone marrow model presented in this study represents a valuable tool for evaluating the efficacy of leukemia therapeutics in a more relevant fashion than commonly used suspension cultures or xenotransplantation of leukemia cells into immunocompromised mice without human stroma support. In the huBM/NSG model, human-derived MSCs generate the stromal microenvironment, both cellular (osteoblasts and mesenchymal cells) and non-cellular (cytokines and ECM proteins secreted by MSCs and their differentiated progeny) needed for support of LSCs. Given the difficulty of generating human-like disease in mice transplanted with primary CLL cells and other types of leukemia, the huBM/NSG model is an improved preclinical model to those currently in use. Utilizing this model will enhance our ability to evaluate the effects of the bone marrow microenvironment on the efficacy of novel leukemia therapeutics, specifically those that reduce LSCs.

In order to avoid the relapsing/remitting course of leukemia, the next generation of anti-leukemia therapeutics needs to eliminate LSCs by, for example, induction of cellular stress or by inhibiting pathways critical for survival of these cells. Such therapeutics must also overcome the environmentally mediated drug-resistance. A previous study from our group demonstrated that compound **3** exerts antiproliferative activity against human leukemia, multiple myeloma, breast, and prostate cancer cell lines *in vitro*.²¹ Here, we show evidence that **3** is potentially capable of reducing primary ALL, AML, and CLL LSCs at micromolar concentrations and may also contribute to induction of oxidative stress. The observation that agent **3** induces cellular stress in primary leukemia cells by itself advocates further investigation of this drug as a novel therapeutic candidate for eliminating LSCs. In addition, other molecules, displaying a similar enone, have been shown to mediate anti-LSC activities via inhibition of NF-kappaB.⁸ Moreover, it is possible that **3** may also be able to mediate such effect. Taken together, these observations provide a strong rationale for further investigating the mechanism of action and the therapeutic potential of compound **3** against leukemia.

More importantly, treatment of mice with **3** led to reduced engraftment of leukemia cells in our humanized bone marrow leukemia xenograft model. Lower numbers of LSCs found in the bone marrow of these animals and additional studies in solid tumors (unpublished data) suggest that the activity of **3** may not be affected by the protective effects mediated by the humanized bone marrow microenvironment. However, further studies in which the compound is injected directly into xenografted mice are needed to confirm this hypothesis. Taken together, our work suggests that compounds **3** is a novel therapeutic candidate that reduces LSCs across a broad range of hematological malignancies. These findings provide a strong rationale for further investigating the therapeutic potential of the semisynthetic alkaloid **3** against LSCs.

Experimental Section

Chemistry

The compounds, (–)-eburnamonine (**1**) and (+)-vincamine (**2**) were supplied by Enzo Life Science (Farmingdale, NY) and Sigma-Aldrich (St. Louis, MO), respectively. The synthesis of compounds **3** was conducted according to the literature procedures.²¹

Primary cells and cell lines

Primary human AML, CLL, and ALL cells, were a kind gift from Dr. Craig Jordan (University of Rochester, NY). Primary cells were obtained after Institutional Review Board approval from the University of Rochester and Purdue University and written informed consent obtained in accordance with the Declaration of Helsinki. Cells were stored in liquid nitrogen, and cells were thawed at 37 °C under 5% CO₂ atmosphere 24 hours prior to use. Cells were cultured in RPMI-1640 (Sigma, St. Louis, MO) media supplemented with 20% normal human serum (Sigma, St. Louis, MO) and 1% penicillin/streptomycin (Sigma, St. Louis, MO) in a humidified 5% CO₂ atmosphere. hTERT-MSCs (human telomerase immortalized human BM mesenchymal stem cell line) were a gift from Dr. Carlotta Glackin (Beckman Research Institute, City of Hope National Medical Center).³⁴ hTERT-MSCs were cultured in α -MEM Eagle media (Sigma, St. Louis, MO) with 10% FBS, 1% penicillin/streptomycin (Sigma, St. Louis, MO) and 1% L-glutamine (Sigma, St. Louis, MO) in a humidified 5% CO₂ atmosphere.

Mice

NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice were purchased from the Jackson Laboratories (Bar Harbor, ME). Mice were bred and housed in a specific pathogen-free environment in ventilated positive air flow racks. Animals were fed irradiated Teklad Global 2918 rodent diet and acidified water (1% acetic acid) *ad libitum*. Animal experiments were performed after approval from the Purdue University's Animal Care and Use Committee and distress during the transplant procedures was minimized by the use of inhalant anesthesia (isoflurane). Animals were monitored daily by the investigators for 3 days following cell transplant. Thereafter, the animals were monitored daily by animal facility staff and twice a week by the investigators or whenever the animal facility staff observed a change in behavior. Unexpected mortality was avoided by euthanizing animals when they first exhibited signs of clinical distress. Animals were euthanized upon signs of disease utilizing the following humane endpoints: tumor size exceeding 1.5 cm³, weight loss, skeletal abnormalities, such as hunched back, failure to thrive, or labored breathing. Animals were euthanized by carbon dioxide asphyxiation followed by cervical dislocation.

Cell transplantation

AML, ALL, and CLL cells (1×10⁵ or 1×10⁶ cells) in 100 μ L of HBSS (Sigma, St. Louis, MO) were injected into the left tibias of male and female mice (8–12 weeks old). Humanization of mouse tibias was performed by co-injecting leukemic cells with 0.25×10⁶ hTERT-MSC bone marrow stromal cells in 100 μ L of HBSS.

Drug treatments

A 10 mM stock of test compound was prepared by dissolving the agent directly in DMSO. For *in vitro* assays, 1×10^6 cells (AML, ALL, and CML) were plated in 1 mL RPMI-1640 media supplemented with 20% normal human serum in a 24-well plate and treated with varying concentrations of test compound (15 μ M, 30 μ M or 50 μ M) or the vehicle control (0.5% DMSO). Viability was assessed 24, 48 or 72 hours following treatment. For *in vivo* assays, 10×10^6 AML, ALL, and CML cells were treated with 30 μ M test compound or 0.3% DMSO in RPMI-1640 media for 24 hours prior to transplantation.

Cell viability assay

Cell viability was determined using the LIVE/DEAD[®] Viability/Cytotoxicity Kit, for mammalian cells (Life technologies, Grand Island, NY). Briefly, following treatment with test compound, cells were washed with $1 \times$ PBS and stained with 1 μ M of calcein-AM and 1 μ M of ethidium at 37 °C for 30 minutes. Images of stained cells were acquired on a Zeiss AxioObserver fluorescent microscope (Zeiss). Image analysis was performed using Zeiss AxioVision (release 4.7.1) software (Zeiss).

Flow cytometry

To determine the effect on LSCs, primary AML, ALL, or CLL cells were cultured in the presence or absence of test compound, washed with $1 \times$ PBS and stained with CD34-APC and either CD38-FITC or CD10-FITC (Beckman Coulter, Brea, CA) on ice for 1 hour. All antibodies were used at a final concentration of 200 ng/mL. Stained cells were analyzed on a Beckman Coulter FC500 flow cytometer. Phenotypically described stem cell subpopulations were quantified by gating the CD34⁺CD38⁻ population for AML²⁴ and CLL²⁵ and the CD34⁺CD10⁻ population for ALL.⁶ To determine human cell engraftment and the effect of test compound on stem cell populations *in vivo*, mice were euthanized 8 weeks post transplantation. Bone marrow (BM) cells were isolated by flushing femurs and tibias of the mice with $1 \times$ PBS. BM cells were stained with antibodies against HLA (200 ng/mL, BD Biosciences, San Jose, CA), CD34, CD38 and CD10.

Statistical analyses

Statistical analysis was performed using the GraphPad Prism 5.0 software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Structures of the natural products, (-)-eburnamonine (1) and (+)-vincamine (2), the semisynthetic derivative, (-)-15-methylene-eburnamonine (3), and the natural products parthenolide (4) and andrographolide (5).

Figure 2.

Biological evaluation of **3** in primary leukemia cells. a) Representative images of AML cells treated with the indicated doses of **3** for 72 hours and stained with calcein AM (green) and ethidium bromide (orange). Summary of viability of primary b) AML, c) ALL, and d) CLL cells in response to **3** (n = 3 per group and data represented mean $\pm$ SD). NT is not treated.

Figure 3.

Summary of fluorescence-activated cell sorting analysis of a) CD34⁺CD38⁻ cells in primary AML samples and b) CD34⁺CD10⁻ cells in primary ALL samples treated with the indicated doses of **3** for 72 hours. Percentages are relative to untreated controls (n = 3 and data represent the mean $\pm$ SD).

Figure 4.

Cytotoxicity studies in primary cells with **3** in the presence or absence of glutathione (GSH). a) Representative images of AML cells treated with 50 μ M of **3** for 72 hours with 500 μ M of GSH and stained with calcein AM (green) and ethidium bromide (orange). Summary of viability studies of primary b) AML and c) ALL cells treated with 50 μ M of **3** for 72 hours with 500 μ M of GSH. ** Indicates $p < 0.01$ and *** indicates $p < 0.001$ on a two tailed t-test.

Figure 5.

Gross images of mice co-injected with hTERT MSCs and primary AML, ALL, and CLL cells pretreated with either the vehicle control (DMSO) or 30 μM of **3** for 24 hours. a) Mice injected with AML cells, and b) spleens with indicated pretreatments. c) Mice injected with ALL cells, and d) cervical lymph nodes with indicated pretreatments. e) Mice injected with CLL cells with indicated pretreatments.

Figure 6.

Summary of fluorescence-activated cell sorting analysis of a) CD34⁺CD38⁻ cells in bone marrow of mice injected with primary AML cells, b) CD34⁺CD10⁻ cells in bone marrow of mice injected with primary ALL cells, and c) CD34⁺CD38⁻ cells in bone marrow of mice injected with primary CLL cells. Cells were treated with DMSO or 30 μM of **3** and mice were euthanized eight-weeks post-transplantation. Cells from three separate patients were injected into three mice each per group. Data from all mice were pooled and are summarized for each disease. Percentages are relative to untreated controls and represent the mean ± SD. *** indicates p = 0.001 on a two tailed t-test.

Table 1

Biological evaluation of **1** and **3** in MDA-MB-231 (breast cancer),²⁰ LNCaP (prostate cancer), RPMI-8226 (myeloma), U266 (myeloma), and KMS-12BM (myeloma) cells.²¹

Cell line	LC ₅₀ (μM) [a]	
	1	3
MDA-MB-213	41.8 (30–58)	14.1 (10–17)
LNCaP	>100	9.5 (7.9–11.4)
RPMI-8226	>100	12.2 (9.3–16.0)
U266	>100	4.3 (3.1–6.0)
KMS-12BM	>100	4.0 (3.3–4.8)

[a] Values are shown with 95% confidence intervals.