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Asunto: Radicación recurso de apelación
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Estimados Señores:

Conforme a la Resolución 42847 del 18 de julio de 2012, me permito adjuntar recurso de reposición en forma oportuna, en contra de la Resolución No. 37571 del 24 de junio de 2013, por medio de la cual se negó la Solicitud de Patente de Invención denominada: "COMPUESTOS DE ESTER BORONATO Y COMPOSICIONES FARMACEUTICAS DE LOS MISMOS" a nombre de MILLENNIUM PHARMACEUTICALS, INC. Solicito que los presentes documentos sean anexados al expediente 10.161.489.

Un cordial saludo.

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

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 La sostenibilidad del medio ambiente comienza en casa, piense antes de imprimir!

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Señores

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

Dirección de Nuevas Creaciones

E. S. D.

REF. PCT- PATENTE DE INVENCION

Invención: COMPUESTOS DE ESTER BORONATO Y COMPOSICIONES
FARMACÉUTICAS DE LOS MISMOS

Expediente: 10.161.489

TITULAR: MILLENNIUM PHARMACEUTICALS, INC.

Asunto: RECURSO DE REPOSICIÓN

MAURICIO PATIÑO BONNET, mayor de edad, vecino de esta ciudad, identificado como aparece al pie de mi firma, en mi calidad de apoderado de la sociedad **MILLENNIUM PHARMACEUTICALS, INC.**, por medio del presente memorial y dentro del término legal correspondiente, presento **RECURSO DE REPOSICIÓN** en contra de la Resolución No. **37571** del 24 de junio de 2013, por la cual se negó la patente de invención de la referencia, para que la revoque y en su lugar se conceda el privilegio de patente solicitado.

Sirven de fundamento los siguientes hechos y argumentos:

HECHOS

- La sociedad **MILLENNIUM PHARMACEUTICALS, INC.**, mediante escrito radicado el 23 de diciembre de 2010, con el número 10.161.489, solicitó la entrada a fase nacional de la solicitud de patente de invención titulada "COMPUESTOS DE ESTER BORONATO Y COMPOSICIONES FARMACÉUTICAS DE LOS MISMOS" que corresponde a la solicitud Internacional PCT/US2009/003602 del 16 de junio de 2009.

- Realizado el examen de forma, se ordenó publicar el extracto de la solicitud en la Gaceta de la Propiedad Industrial No. 632, sin que se hubieran presentado oposiciones por parte de terceros.
- Realizado el examen de fondo, se requirió al solicitante mediante Oficio No. **463**, notificado el 22 de enero de 2013, para que se pronunciara sobre la patentabilidad de la invención en términos de claridad, excepciones de patentabilidad, novedad y nivel inventivo.
- El solicitante respondió oportunamente los requerimientos realizados, mediante escrito radicado el 07 de junio de 2013, y presentó un nuevo capítulo reivindicatorio compuesto por diecisiete (17) reivindicaciones.
- Mediante Resolución No. **37571** del 24 de junio de 2013, la Superintendencia de Industria y Comercio, negó la patente de invención solicitada.

FUNDAMENTOS DE LA DIRECCIÓN

La Dirección fundamenta la decisión sosteniendo lo siguiente:

Como se dijo anteriormente, el documento D1 es considerado el estado de la técnica cercano a la invención definida en la reivindicación 1 porque divulga un compuesto de fórmula (1) y composiciones que los contienen con actividad inhibitoria de proteasoma (Pág. 1, Párr. [0011] a Pág. 2, Párr. (0020)).

La diferencia entre la invención, definida en la reivindicación 1, y los compuestos del documento D1 consiste en que este documento no divulga específicamente el anillo 2,5-diclorofenilo unido a un sustituyente carbonilo como grupo P y no divulga específicamente la estructura derivada de dioxaborolano o dioxaborinano oxo sustituida comprendiendo los sustituyentes derivados de ácido carboxílico R^{b3} y R^{b4} . En los ejemplos del documento D1 se puede observar que en este documento ya se encuentra divulgado el grupo isobutilo como sustituyente correspondiente al grupo R^a del compuesto reclamado.

El efecto que logra el incluir el grupo 2,5-diclorofenilcarboxamida y los anillos dioxaborolano y dioxaborinano oxosustituidos comprendiendo los sustituyentes derivados de ácido carboxílico R^{b3} y R^{b4} es que permite obtener compuestos alternativos como inhibidores de proteasoma.

El problema técnico objetivo que pretende resolver esta invención se puede formular así: ¿Cómo modificar los compuestos conocidos en D1 para proporcionar compuestos alternativos como inhibidores de proteasoma?

Ahora bien, teniendo en cuenta que el documento D1 da a conocer compuestos de fórmula (I) inhibidores de proteasoma que tienen un anillo aromático unido a un grupo carboxamida y comprenden estructuras cíclicas como son los anillos dioxaborolano y dioxaborinano; se puede observar que el anillo 2,5-diclorofenilo y los sustituyentes R^{b3} y R^{b4} así como el sustituyente oxo de los anillos dioxaborolano y dioxaborinano son las diferencias estructurales entre los compuestos reclamados y los compuestos conocidos según el documento D1. Pese a esta diferencia no se presenta evidencia de un efecto técnico sorprendente otorgado al compuesto al seleccionar específicamente el anillo 2,5-diclorofenilo como anillo aromático y al incluir sustituyentes oxo y $-(CH_2)_p-CO_2H$ como sustituyentes de los anillos dioxaborolano y dioxaborinano. De esta manera esta oficina concluye que una persona normalmente versada en la materia seleccionaría el anillo de fenilo como anillo correspondiente al sustituyente R^7 divulgado en dicho documento y realizaría modificaciones menores sobre este anillo de fenilo y sobre los anillos de dioxaborolano y dioxaborinano para llegar al objeto de la reivindicación 1, por lo cual y ante la falta de evidencia de un efecto técnico sorprendente presentado por el compuesto reclamado, dicha reivindicación se considera obvia.

Lo anterior considerando que la actividad se debe al núcleo farmacofórico de la molécula y la modificación realizada en la estructura, que es una modificación menor, no ocasiona una variación importante en su actividad, lo cual es considerado obvio y por lo tanto, sin altura inventiva.

Puesto que para los demás compuestos reclamados así como para las formas cristalinas y las composiciones reivindicadas en la solicitud, no se presenta evidencia de un efecto técnico sorprendente; se considera que las reivindicaciones 2 a 17 tampoco tienen nivel inventivo.

La solicitante afirma que el documento D1 especifica que el termino radical derivado de azúcar se refiere a un radical formado por la remoción de átomos de hidrogeno desde dos grupos hidroxilo de cualquier radical de azúcar y que ni el ácido pirúvico ni el ácido cítrico corresponden a dicho tipo de radical, estableciendo además que la solicitud especifica que los radicales utilizados se refieren a un grupo radical directamente conectado a un átomo de carbono en una posición alfa o beta relativa al grupo ácido carboxílico y que por lo tanto no hay ninguna enseñanza en D1 que pueda conducir a llegar a los compuestos reivindicados de la fórmula (II) sin un descubrimiento inventivo.

Adicionalmente la solicitante manifiesta que "las nuevas reivindicaciones 6 a 14 relacionadas con nuevas formas cristalinas caracterizadas por patrones de difracción de polvo de rayos X con picos característicos, muestran propiedades distintivas en la unidad de empaque, termodinámica y temperatura de fusión" argumentando que el documento D1 no proporciona enseñanzas o sugerencias que guíen a obtener dichas nuevas formas cristalinas.

Se aclara a la solicitante que el documento D1 sugiere todas las características técnicas del grupo farmacofórico principal incluyendo el anillo 2,5-diclorofenilo el cual queda sugerido cuando en dicho documento se establece que el sustituyente R⁷ corresponde a un anillo sustituido (Pág. 5, Párr. [0081]). Si bien el documento D1 no proporciona enseñanzas que permitan llegar a la consecución de los restos de ácido pirúvico y cítrico comprendiendo los sustituyentes oxo y $-(CH_2)_p-CO_2H$ como sustituyentes de los anillos dioxaborolano y dioxaborinano, estos constituyen una modificación menor sobre el grupo farmacofórico principal dentro de la experimentación para obtener compuestos alternativos como inhibidores de proteasoma y puesto que la solicitante no presenta evidencia que bajo las mismas condiciones los compuestos y las formas cristalinas de la presente invención presenten un efecto técnico sorprendente con respecto a los compuestos ya conocidos en el estado de la técnica. De manera que el nivel de actividad de dichos compuestos y formas cristalinas de la solicitud estudiada no es sorprendente para la persona normalmente conocedora de la materia, a la luz de lo enseñado en el documento D1.

ARGUMENTOS DEL RECURSO

En primer lugar deseo expresar mi total desacuerdo con las razones expuestas por ese Despacho respecto a la falta de nivel inventivo de la presente invención. Cabe resaltar los siguientes puntos que claramente demuestran que la presente invención cumple con el nivel inventivo necesario para que le sea concedido el privilegio de patente de invención. A saber:

Establece el artículo 18:

"Art. 18

Se considerará que una invención tiene nivel inventivo, si para una persona del oficio normalmente versada en la materia técnica correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica".

En este sentido es importante poner de presente que el examen de nivel inventivo está dirigido a determinar si la invención reivindicada había sido sugerida o enseñada en el estado del arte, de acuerdo con el conocimiento de un técnico medio en la materia. Para los anteriores efectos, el método problema-solución le indica al Examinador que al realizar el examen de nivel inventivo debe:

"- Identificar el estado de la técnica cercano a la invención reivindicada

El estado de la técnica cercano es un documento que se encuentra en el mismo campo técnico que la invención o trata de solucionar el mismo problema o uno semejante. Suele ser el que tiene más características en común con la invención o presenta leves modificaciones de estructura e indica la misma función, propósito. En caso de que haya varios documentos relacionados, el estado de la técnica cercano es el documento que menciona una función, propósito, problema a resolver ó actividad semejante al de la invención.

-Determinar la diferencia entre la invención y estado de la técnica cercano.

- Definir el efecto técnico causado y atribuible al elemento diferencial.

- Deducir el problema técnico objetivo.

- *Evaluar si la invención reivindicada, partiendo del estado de la técnica cercano y del problema técnico objetivo, habría sido obvia para la persona medianamente versada en la materia."*¹

En este sentido, el instructivo para el Examen de Solicitudes de Patentes de Invención y Modelos de Utilidad de la Superintendencia de Industria y Comercio, establece:

*"Si un compuesto tiene estructura similar a un compuesto conocido y su efecto es sorprendente, se considera inventivo"*².

El Despacho considera que la invención de mi representada carece de nivel inventivo a la luz de **D1** (US2002188100) porque no se evidencia un efecto técnico sorprendente al seleccionar específicamente el anillo 2,5-diclorofenilo como anillo aromático y al incluir sustituyentes oxo y $-(CH_2)_p-CO_2H$ como sustituyentes de los anillos dioxaborolano y dioxaborinano.

En vista de lo anterior, presento junto con este escrito un artículo de *Erik Kupperman, et al.*, el cual fue publicado por la AACR – *American Association for Cancer Research*, en la edición del 1 de marzo de 2010 titulado *"Evaluación del inhibidor de proteasoma MLN9708 en Modelos Preclínicos de cáncer humano"*. Dicho artículo compara la efectividad del compuesto MLN9708, objeto de protección de la presente solicitud de patente, con el compuesto bortezomib; compuesto revelado y ejemplificado en el Ejemplo 2, pág. 10 de **D1**, identificado por el examinador como el estado de la técnica más cercano.

En este respecto, el artículo afirma:

"MLN9708 tiene una vida media de disociación de proteasoma más corta, así como farmacocinética, farmacodinámica y actividad antitumoral mejorada en comparación con el bortezomib. MLN9708 tiene una mayor distribución de volumen de sangre en estado estable, y los análisis de la inhibición de la proteasoma 20S, así como los marcadores de la respuesta de proteína desplegada confirmaron que MLN9708 tiene mayores efectos

¹ Superintendencia de Industria y Comercio **"Examen de Solicitudes de Patentes de Invención y Modelos de Utilidad"**, Bogotá D.C., Febrero 8, 2012, Pág. 72 - 73.

² *Ibidem*, Pág. 85.

farmacodinámicos en los tejidos a comparación con el bortezomib” (Ver el Resumen).

“MLN9708 tiene una vida media de disociación de proteasoma más corta que el bortezomib, lo cual creemos que juega un rol importante en la mejoría en la distribución de tejidos. La comparación directa con el bortezomib reveló que MLN9708 tiene un perfil farmacocinético y farmacodinámico mejorado y muestra una actividad antitumoral superior tanto en tumores sólidos como en modelos de xenoinjertos hematológicos, y muestra actividad antitumoral cuando es administrado a través de múltiples rutas de dosificación y regímenes” (ver el final de la sección de la Introducción).

“Una superior biodisponibilidad igualmente permite que esta molécula sea administrada de forma oral, mientras que el bortezomib se limita a regímenes de administración por vía intravenosa y vía subcutánea actividad antitumoral” (Ver el último párrafo).

Por consiguiente, toda vez que las diferencias estructurales de los compuestos de la presente invención: (i) no son sugeridas o reveladas en **D1** y (ii) dichas diferencias **SÍ permiten obtener un efecto técnico sorprendente, evidenciado en una biodisponibilidad superior, un perfil farmacocinético y farmacodinámico mejorado, y una mejor actividad antitumoral**; se puede concluir, que **D1** no anticipa la materia de la presente invención y por lo tanto los compuestos y las formas cristalinas aquí reivindicados son inventivos a la luz de la anterioridad citada.

PETICIÓN

Atentamente solicito a su Despacho se sirva revocar la Resolución No. **37571** del 24 de junio de 2013, por la cual se negó la solicitud patente de la referencia y en su lugar, se conceda el privilegio de patente a la invención “COMPUESTOS DE ESTER BORONATO Y COMPOSICIONES FARMACÉUTICAS DE LOS MISMOS” a nombre de **MILLENNIUM PHARMACEUTICALS, INC.**, contenida en el Expediente No. 10.161.489.

PRUEBAS

Comendidamente solicito tener en cuenta como prueba de las argumentaciones expuestas en el presente recurso, el material constitutivo del Expediente No. 10.161.489 referente a la patente en discusión.

NOTIFICACIONES

Recibiré notificaciones en la secretaría de su Despacho o en la Carrera 9 # 74-08, Oficina 305, en la ciudad de Bogotá D.C.

ANEXOS

- i) Erik Kupperman, Edmund C. Lee, Yueying Cao, et al. "*Evaluation of the Proteasome Inhibitor MLN9708 in Preclinical Models of Human Cancer*". *Cancer Res* 2010;70:1970-1980. February 16, 2010, junto con su respectiva traducción al español.

Señor Jefe de la Dirección de Nuevas Creaciones,

prietocarrizosa



MAURICIO PATIÑO BONNET
C.C. 11.342.699 de Zipaquirá
T.P. 73.583 del C.S. de la J.



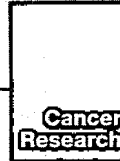
Cancer Research

Evaluation of the Proteasome Inhibitor MLN9708 in Preclinical Models of Human Cancer

Erik Kupperman, Edmund C. Lee, Yueying Cao, et al.

Cancer Res 2010;70:1970-1980. Published OnlineFirst February 16, 2010.

Updated version	Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-09-2766
Supplementary Material	Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2010/02/15/0008-5472.CAN-09-2766.DC1.html
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Evaluation of the Proteasome Inhibitor MLN9708 in Preclinical Models of Human Cancer

Erik Kupperman, Edmund C. Lee, Yueying Cao, Bret Bannerman, Michael Fitzgerald, Allison Berger, Jie Yu, Yu Yang, Paul Hales, Frank Bruzzese, Jane Liu, Jonathan Blank, Khristofer Garcia, Christopher Tsu, Larry Dick, Paul Fleming, Li Yu, Mark Manfredi, Mark Rolfe, and Joe Bolen

Abstract

The proteasome was validated as an oncology target following the clinical success of VELCADE (bortezomib) for injection for the treatment of multiple myeloma and recurring mantle cell lymphoma. Consequently, several groups are pursuing the development of additional small-molecule proteasome inhibitors for both hematologic and solid tumor indications. Here, we describe MLN9708, a selective, orally bioavailable, second-generation proteasome inhibitor that is in phase I clinical development. MLN9708 has a shorter proteasome dissociation half-life and improved pharmacokinetics, pharmacodynamics, and antitumor activity compared with bortezomib. MLN9708 has a larger blood volume distribution at steady state, and analysis of 20S proteasome inhibition and markers of the unfolded protein response confirmed that MLN9708 has greater pharmacodynamic effects in tissues than bortezomib. MLN9708 showed activity in both solid tumor and hematologic preclinical xenograft models, and we found a correlation between greater pharmacodynamic responses and improved antitumor activity. Moreover, antitumor activity was shown via multiple dosing routes, including oral gavage. Taken together, these data support the clinical development of MLN9708 for both hematologic and solid tumor indications. *Cancer Res*; 70(5): 1970–80. ©2010 AACR.

Introduction

The ubiquitin-proteasome system processes the majority of cellular proteins and is the principal manner by which cells regulate protein homeostasis. During normal protein homeostasis, specific proteins are targeted for destruction via the attachment of ubiquitin. These proteasome substrates include misfolded proteins and highly regulated members of critical signaling cascades, including proteins involved in growth control, cell cycle regulation, and apoptosis. Proteasome inhibition results in the stabilization and accumulation of these substrates, leading to the activation of antiproliferative signals, cell cycle disruption, activation of apoptotic pathways, and, ultimately, cell death (1, 2). Rapidly growing malignant cells, already deficient in normal cell cycle checkpoint mechanisms, seem to be highly susceptible to proteasome inhibition (3–6). Therefore, the proteasome emerged as an attractive target for anticancer

therapeutics. The success of the first-in-class small-molecule proteasome inhibitor VELCADE (bortezomib) for injection (Millennium Pharmaceuticals, Inc.) validated the proteasome as a therapeutic target for the treatment of human cancer (1, 7–12). VELCADE is approved for the treatment of patients with multiple myeloma and previously treated mantle cell lymphoma (13–21). At present, there are multiple groups in the process of developing small-molecule proteasome inhibitors for various oncology indications. These include both reversible inhibitors, such as CEP-18770, and irreversible inhibitors, such as carfilzomib and NPI-0052. Both CEP-18770 and NPI-0052 are orally active, and all three compounds have shown antitumor activity in preclinical models and are currently in various stages of clinical development (22–30).

The 26S proteasome consists of a 20S multicatalytic core capped on either end with 19S regulatory subunits. The 20S proteasome is a chambered, barrel-like structure containing two heptameric rings made from α subunits and two heptameric rings made from β subunits. The α rings perform capping and gating functions, whereas three of the β subunits ($\beta 1$, $\beta 2$, and $\beta 5$) contain the NH_2 -terminal threonines responsible for the different proteasome proteolytic activities. The $\beta 1$, $\beta 2$, and $\beta 5$ subunits are referred to as caspase-like, trypsin-like, and chymotrypsin-like, respectively, because the preferred cleavage site of each subunit is similar to those of other well-known proteases (2, 31–34).

Bortezomib shows time-dependent inhibition of the 20S proteasome by binding to the NH_2 -terminal threonine side chain of the catalytic β subunits. Bortezomib exhibits

Authors' Affiliation: Millennium Pharmaceuticals, Inc., Cambridge, Massachusetts

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

E. Kupperman and E.C. Lee contributed equally to this work.

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inhibitory activity against all three β subunits but preferentially binds to and inhibits the $\beta 5$ site (35). Although bortezomib has shown clinical efficacy in multiple myeloma and mantle cell lymphoma, to date, it has yet to exhibit strong activity in solid tumor indications, perhaps due to its inability to penetrate into tissues and achieve therapeutically relevant concentrations at those target sites. Therefore, there is a strong rationale for identifying proteasome inhibitors that have different physicochemical or pharmacokinetic properties. Here, we describe the biochemical and preclinical pharmacology data that support the development of MLN9708. MLN9708 is a second-generation small-molecule proteasome inhibitor being developed for the treatment of a broad range of human malignancies. MLN9708 was selected from a large pool of boron-containing proteasome inhibitors based on a physicochemical profile that was distinct from bortezomib. MLN9708 has a shorter 20S proteasome dissociation half-life than bortezomib, which we believe plays an important role in its improved tissue distribution. Direct comparison with bortezomib revealed that MLN9708 has an improved pharmacokinetic and pharmacodynamic profile and shows superior antitumor activity in both solid tumor and hematologic xenograft models, and shows antitumor activity when administered via multiple dosing routes and regimens. MLN9708 is currently being evaluated in multiple phase I clinical studies for both solid- and hematologic-based tumors.

Materials and Methods

Cell Culture

WSU-DLCL2, OCI-Ly7, A375, H460, HCT-116, HT-29, MDA-MB-231, HEK293, and Calu-6 cells were obtained from the American Type Culture Collection and maintained as recommended by the supplier.

In vitro Assays

Kinetic analysis of 20S proteasome inhibition. Kinetic analysis of 20S proteasome inhibition was performed as previously described by Williamson and colleagues (36).

NF- κ B-Luc and 4 $\times$ Ub-Luc cell-based reporter assays. NF- κ B-Luc and 4 $\times$ Ub-Luc cell-based reporter assays were performed as previously described by Williamson and colleagues (36).

Proteasome-Glo IC₅₀ and inhibitor washout cell-based assays. Calu-6 cells were cultured in MEM containing 10% fetal bovine serum and 1% penicillin/streptomycin and plated 1 d before the start of the experiment at 10,000 cells per well in a 384-well plate. For IC₅₀ determinations, cells were treated with varying concentrations of bortezomib or MLN2238 in DMSO (0.5% final, v/v) for 1 h at 37°C. For reversibility experiments, cells were treated with either 1 μ M bortezomib or MLN2238 for 30 min at 37°C and then washed thrice in medium to remove the compounds. Cells were incubated for an additional 4 h at 37°C, after which the medium was removed and replaced with fresh medium. Proteasome activity was assessed by monitoring hydrolysis of the chymo-

trypsin-like substrate Suc-LLVY-aminoluciferin in the presence of luciferase using the Proteasome-Glo assay reagents according to the manufacturer's instructions (Promega Corp.). Luminescence was measured using a LEADseeker instrument (GE Healthcare Life Sciences).

Pharmacokinetic Studies

Blood and tumor samples were collected before dose and numerous time points after dosing. Each time point represents the average value of three animals. MLN2238 or bortezomib concentrations in blood and plasma samples were determined using a non-good laboratory practice liquid chromatography-tandem mass spectrometry (LC/MS/MS)-based method. MLN2238 or bortezomib was isolated from 50 μ L of plasma or blood using a liquid-liquid extraction procedure. Sample (50 μ L) was mixed with 50 μ L of internal standard solution, 50 μ L of 0.5 mol/L HCl, and 500 μ L of methyl tertiary butyl ether. The supernatant (300 μ L) was then transferred to a clean 96-well plate, evaporated, reconstituted in 100 μ L of acetonitrile/water (5:95) containing 0.1% formic acid, and injected onto the LC/MS/MS system for analysis. A reverse-phase gradient method provided sample stacking and separation. Pharmacokinetic analysis of the blood and plasma concentration data was performed using WinNonlin version 5.2 (Pharsight Corp.). Kinetic parameters were estimated using a noncompartmental model using sparse sampling mode (model 201 for plasma and blood). Area under the concentration versus time curve (AUC) and area under the effect versus time curve (AUE) values were calculated using the linear trapezoidal rule.

Pharmacodynamic Studies

Approximately 200 μ L of whole blood were collected from each animal and processed for the 20S blood proteasome inhibition assay. Subcutaneous tumors (approximately 600–800 mm³ in size) were harvested and divided into two or three parts. One was processed for the 20S tissue proteasome inhibition assay, one for Western blot analysis, and one for immunohistochemistry.

Tumor processing for 20S tissue proteasome assays. Frozen samples were pulverized in the Tissue CryoPrep (Covaris) and transferred to glass tubes. After addition of 1 mL of cold tissue lysis buffer [50 mmol/L HEPES (pH 8.0), 1 mmol/L DTT], samples were placed on ice and homogenized as per the manufacturer's instructions using the Covaris E200.

Tumor processing for Western blot assays. Tumors were processed as described above in the Covaris E200. M-PER lysis buffer (Pierce) was supplemented with the following: 1 $\times$ protease inhibitor cocktail set (Calbiochem), 2 mmol/L sodium orthovanadate (Sigma), 25 mmol/L sodium fluoride, and 25 mmol/L β -glycerophosphate. Cold lysis buffer (300–800 μ L) was added to the tumors just before sonication. After sonication, supernatants were transferred to new tubes and protein concentrations were determined.

Western blot analysis. Tumor lysate (50 μ g) was loaded onto 4% to 12% Bis-Tris gels (Invitrogen). Proteins were transferred to PVDF-FL membranes (Millipore) using a

semidry transfer apparatus. After transfer and blocking, membranes were incubated with primary antibody overnight at 4°C. Membranes were washed thrice with TBS-Tween 20 (TBST) and incubated with Alexa Fluor 680-labeled goat anti-rabbit immunoglobulin G (Molecular Probes) for 1 h. Membranes were washed five times with TBST and once with TBS while protected from light. Membranes were dried and scanned with the Odyssey Infrared Imaging System (LI-COR Biosciences). The following primary antibodies were used: anti-tubulin (rabbit polyclonal, 1:15,000 dilution; Abcam) and anti-growth arrest DNA damage 34 (GADD34; Proteintech Group, Inc.). Secondary antibody was used at 1:20,000 for tubulin and 1:2,000 for GADD34. Quantitation of Western blot signals was performed with Odyssey software.

20S β 5 proteasome tumor and blood assays. 20S β 5 proteasome tumor and blood assays were performed as previously described (36–38).

Immunohistochemical studies. Formalin-fixed, paraffin-embedded CWR22 and WSU-DLCL2 xenograft tumor sections (5 μ m) were stained with primary antibodies to GADD34, activating transcription factor 3 (ATF3), and cleaved caspase-3 (Proteintech Group, Santa Cruz Biotechnology, and Cell Signaling Technology). The GADD34 and ATF3 antibodies were detected with horseradish peroxidase-labeled secondary antibodies (UltraMap anti-rabbit, Ventana Medical Systems) and incubated with the Chromo-Map 3,3'-diaminobenzidine (DAB) kit (Ventana Medical Systems). The cleaved caspase-3 antibody was detected with Alexa Fluor 594-labeled secondary antibody (Invitrogen). Slides were counterstained with hematoxylin for GADD34 and ATF3 assays and 4',6-diamidino-2-phenylindole for cleaved caspase-3 assay. Images were captured using an Eclipse E800 microscope (Nikon Instruments), 20 $\times$ objective, and Retiga EXi color digital camera (QImaging). Five fields of view were captured per sample, and images were processed using MetaMorph software (Molecular Devices). Pharmacodynamic marker levels were measured by color thresholding on the DAB or fluorescent signal and measuring area of thresholded signal. Percent positive area was calculated by normalizing with the total area of the field of view.

Animal Care

CB17–severe combined immunodeficient (SCID) and non-obese diabetic (NOD)–SCID mice were housed and maintained in a controlled environment and received food and water *ad libitum*. Veterinary care for the animals was provided in accordance with Millennium Institutional Animal Care and Use Committee.

Efficacy Studies

CWR22 xenografts. Male CB17-SCID mice (Charles River Laboratories), approximately 8 to 11 wk of age, were inoculated s.c. with freshly dissected CWR22 tumor fragments (~20 mg) in the right dorsal flank. Mean tumor volume (MTV) was calculated using the following formula: $0.5 \times (\text{length} \times \text{width}^2)$. When MTV reached approximately

150 to 200 mm³, animals were randomized into treatment groups ($n = 10$ per group) before dosing. Antitumor activity was determined at the end of the study by calculating the treatment over control (T/C) ratio of their MTVs at the end of the study.

WSU-DLCL2 xenografts. Female CB17-SCID mice, ~6 wk of age, were inoculated s.c. with 4×10^6 WSU-DLCL2 tumor cells suspended in 0.1 mL RPMI 1640 in the right dorsal flank. Animals were randomized, and the MTV and T/C ratio were calculated as described above.

OCI-Ly7-Luc disseminated xenografts. Female NOD-SCID mice, ~9 wk of age, were inoculated i.v. via the tail vein with 1.0×10^6 OCI-Ly7-Luc tumor cells. Mice were randomized into treatment groups ($n = 10$ per group) on day 7 after inoculation. For each imaging session, animals received 150 mg/kg of luciferin (Caliper Life Sciences) via i.p. injection. Animal dorsal and ventral views were imaged to determine total photon flux. Images were captured by the Xenogen IVIS imaging system (Xenogen Corp.), and data were collected with Xenogen Living Image software (Living Image 3.0.2.2). Antitumor activity was determined by calculating the T/C ratio of the mean photon flux measurements at the end of the study. Survival curves were generated using the Kaplan-Meier method.

Statistical Analyses

Efficacy data were analyzed using a linear mixed-effect regression model. Differences among mice were treated as random effects, and a compound symmetry covariance structure was used to model the variability between repeated tumor measurements for each mouse. Treatment comparisons were performed by taking fitted curves from the model to calculate Δ AUCs. The significance of the Δ AUC was assessed using permutation testing. P values of ≤ 0.05 were considered significant. For the OCI-Ly7-Luc study, differences in total photon flux among mice were compared using one-way ANOVA and pairwise comparisons were adjusted by the Tukey-Kramer method. Survival curves generated using the Kaplan-Meier methods were compared using the log-rank (Mantel-Cox) test and pairwise comparisons were adjusted with the Bonferroni correction.

Results and Discussion

MLN2238 is a selective, potent, and reversible inhibitor of the proteasome. MLN9708 was identified in screens for small-molecule proteasome inhibitors with an improved pharmacologic profile compared with bortezomib (Fig. 1). In preclinical studies, MLN9708 immediately hydrolyzed to MLN2238 (see Supplementary Data), the biologically active form, on exposure to aqueous solutions or plasma. In studies where a solution of MLN9708 was added directly into rat, dog, or human plasma and immediately extracted and analyzed by high-performance liquid chromatography, only MLN2238 could be identified. MLN2238 is an N-capped dipeptidyl leucine boronic acid and preferentially bound to and inhibited the chymotrypsin-like proteolytic (β 5) site

of the 20S proteasome with an IC_{50} value of 3.4 nmol/L (K_i of 0.93 nmol/L; Table 1). At higher concentrations, it also inhibited the caspase-like ($\beta 1$) and trypsin-like ($\beta 2$) proteolytic sites (IC_{50} of 31 and 3,500 nmol/L, respectively). Although the selectivity and potency of MLN2238 were similar to that of bortezomib, the proteasome binding kinetics for these two molecules are different. Both MLN2238 and bortezomib showed time-dependent reversible proteasome inhibition; however, the proteasome dissociation half-life ($t_{1/2}$) for MLN2238 was determined to be ~6-fold faster than that of bortezomib ($t_{1/2}$ of 18 and 110 minutes, respectively).

MLN2238 is a potent inhibitor of the proteasome in tumor cells. To build on the biochemistry results, a series of cell-based experiments were performed to confirm potent proteasome inhibition in cells. Proteasome inhibition results in the stabilization and accumulation of ubiquitinated proteins, which have been targeted for destruction.

This leads to cell cycle disruption, activation of apoptotic pathways, and active cell death (39–43). Initial studies examined the effects of MLN2238 treatment on an exogenous proteasome substrate. MDA-MB-231 cells expressing a 4xUb-Luc reporter (36) were treated with increasing concentrations of MLN2238 and bortezomib. Both compounds strongly inhibited proteasome activity, resulting in accumulation of the luciferase reporter with similar EC_{50} values (Table 1). The effect of bortezomib and MLN2238 on tumor necrosis factor- α (TNF- α)-induced activation of the NF- κ B pathway was also examined (44). Proteasome inhibition prevents the degradation of I κ B α , an inhibitor of NF- κ B, resulting in a decrease in NF- κ B-driven gene expression. HEK293 cells stably expressing a NF- κ B-Luc reporter were treated with increasing concentrations of MLN2238 and bortezomib. Both compounds strongly inhibited TNF- α -induced activation of the NF- κ B pathway, resulting in similar EC_{50} values (Table 1).

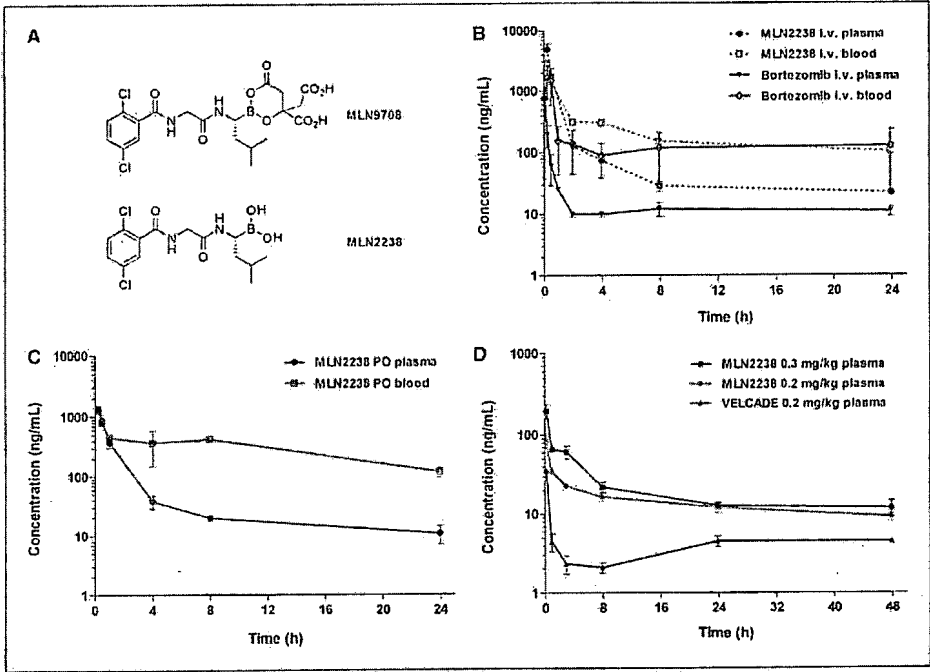


Figure 1. Structure of MLN9708. A, blood and plasma concentration versus time profile of MLN2238 and bortezomib in CB17-SCID mice following an acute i.v. administration (100 μ L per mouse) at 14 or 0.6 mg/kg, respectively. B, blood and plasma concentration versus time profile following an acute oral administration of MLN2238 in CB17-SCID mice at 11 mg/kg (100 μ L per mouse). C, plasma concentration versus time profile following an acute i.v. administration (200 μ L per rat) of MLN2238 and bortezomib in nude rats at 0.3, 0.2, and 0.2 mg/kg, respectively. D, bars, SD, $n = 3$ for all time points, except 0.5 h after dose in plasma for MLN2238 i.v., 1 h after dose in plasma for bortezomib i.v., and 0.5 and 8 h after dose in blood for MLN2238 orally (PO), where $n = 2$.

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Table 1. Summary of MLN2238 and bortezomib enzymology, pharmacokinetic, and pharmacodynamic parameters						
			MLN2238	Bortezomib		
Biochemical assays						
β5 K _i (nmol/L)			0.93 (0.64–1.4, n = 3)			0.55 (0.34–0.89, n = 3)
β5 IC ₅₀ (nmol/L)			3.4 (2.8–4.1, n = 3)			2.4 (2.0–2.9, n = 45)
β2 IC ₅₀ (nmol/L)			3,500			1,200
β1 IC ₅₀ (nmol/L)			31			24 (14.5–40, n = 12)
β5 dissociation half-life (min)			18 (6.8–30, n = 3)			110 (71–150, n = 3)
Cell-based assays						
MDA-MB-231 4xUb-Luc EC ₅₀ (nmol/L)			525 (330–840, n = 4)			310 (230–400, n = 29)
E _{max} (fold stimulation)			265 (160–370, n = 4)			370 (330–410, n = 29)
HEK293 NF-κB-Luc EC ₅₀ (nmol/L)			55 (33–91, n = 7)			33 (27–40, n = 23)
E _{max} (% maximum inhibition)			99.3 (99.0–99.6, n = 7)			99.6 (99.3–100, n = 23)
Calu-6 Proteasome-Glo IC ₅₀ (nmol/L)			9.7 (n = 7)			4.8 (n = 12)
Calu-6 Proteasome-Glo (% activity),* t = 4 h, no washout			7.1 (3.6–10.6, n = 5)			3.45 (2.0–4.9, n = 5)
Calu-6 Proteasome-Glo (% Activity),* t = 4 h, washout			69 (66–71, n = 5)			20 (18–23, n = 5)
A375 ATPite LD ₅₀ (nmol/L)			20			6.5
H460 ATPite LD ₅₀ (nmol/L)			58			13
HCT-116 ATPite LD ₅₀ (nmol/L)			19			4
HT-29 ATPite LD ₅₀ (nmol/L)			52			7
Pharmacokinetic parameters						
Agent	Dose and route	Matrix	C _{max} (ng/mL)	AUC _{0–24h} (h·ng/mL)	Vd (L/kg)	F%
MLN2238	14 mg/kg i.v.	Plasma	17,000	8,090	20.2	27.8
	14 mg/kg i.v.	Blood	10,500	9,660		
	11 mg/kg orally	Plasma	1,630	1,810		
	11 mg/kg orally	Blood	1,710	6,310		
Bortezomib	0.8 mg/kg i.v.	Plasma	321	485	4.3	
	0.8 mg/kg i.v.	Blood	548	4422		
Pharmacodynamic parameters						
Agent	Dose and route	Matrix	E _{max} (I%)	AUE _{0–24h} (%I·h)	AUE ratio (tumor/blood)	
MLN2238	14 mg/kg i.v.	Blood	83.1 [†]	718 [†]		
	14 mg/kg i.v.	Tumor (CWR22)	69.1	1120	1.56	
	14 mg/kg i.v.	Tumor (WSU-DLCL2)	77.0	1460	2.03	
Bortezomib	0.8 mg/kg i.v.	Blood	88.3 [‡]	1170 [‡]		
	0.8 mg/kg i.v.	Tumor (CWR22)	44.8	804	0.69	
	0.8 mg/kg i.v.	Tumor (WSU-DLCL2)	27.6	306	0.26	
NOTE: Results are reported as mean (95% confidence interval, number of experiments).						
Abbreviations: K _i , inhibition dissociation constant; E _{max} , maximum effect; F%, oral bioavailability; t = time; AUC _{0–24 h} , AUC from 0 to 24 h; C _{max} , maximum concentration; Vd, volume of distribution; I%, percentage of inhibition.						
*After exposure to 1 μmol/L MLN2238 or 1 μmol/L bortezomib for 30 min.						
[†] For MLN2238, blood E _{max} = 81.3% to 85.0% (n = 2) and AUE _{0–24h} = 554 to 882 (n = 2).						
[‡] For bortezomib, blood E _{max} = 86.8% to 89.8% (n = 2) and AUE _{0–24h} = 1,140 to 1,200 (n = 2).						

The effect of MLN2238 or bortezomib on β5 activity was determined *in situ* using the Proteasome-Glo cell-based assay. The IC₅₀ values determined by this assay following 1 hour of treatment with MLN2238 or bortezomib were in the low nanomolar range and comparable with those calculated with purified 20S proteasome. Recovery of pro-

teasome activity was determined by performing washout experiments with MLN2238 or bortezomib. Cells were treated with the drug for 4 hours, after which the drug was removed and proteasome activity was assessed. Proteasome activity in MLN2238-treated cells recovered to 69% of control cells, whereas activity in bortezomib-treated

cells recovered to only 20% (Table 1). The difference in recovery of proteasome activity between MLN2238 and bortezomib is consistent with the observed differences in proteasome $t_{1/2}$ between the two molecules.

Cell viability studies were performed in a variety of mammalian cell lines to compare the *in vitro* antiproliferative effects of MLN2238 with bortezomib. Studies performed with A375 (lung), H460 (lung), HCT-116 (colon), and HT-29 (colon) cells revealed similar LD_{50} values for the two compounds, which ranged from 4 to 58 nmol/L (Table 1).

Taken together, these *in vitro* studies show that MLN2238 is a potent inhibitor of the $\beta 5$ site of the 20S proteasome and that MLN2238 dissociated more rapidly from the proteasome than bortezomib, consistent with faster recovery of proteasome activity observed in the Proteasome-Glo assay. Given the high concentrations of proteasome found in RBCs, we hypothesized that RBC partitioning would serve

as a drug sink for bortezomib and limit its distribution outside of the blood compartment; the shorter proteasome $t_{1/2}$ of MLN2238 should allow improved drug distribution into tissues. To address this issue directly, a series of pharmacokinetic, pharmacodynamic, and efficacy studies with MLN2238 and bortezomib were performed in different xenograft models.

Pharmacokinetics of MLN2238 and bortezomib. To determine the pharmacokinetic profile of MLN2238 and bortezomib, mice were administered a single dose of MLN2238 at 14 mg/kg i.v. and 11 mg/kg orally or bortezomib at 0.8 mg/kg i.v. These doses represent the maximum tolerated dose (MTD) for each drug for the specified route of administration. Exposures were determined by measuring the blood and plasma drug concentrations at various time points following the initial dose (Table 1). The concentration-versus-time curve of i.v. administered MLN2238 displayed a distinct biexponential profile with a steep ini-

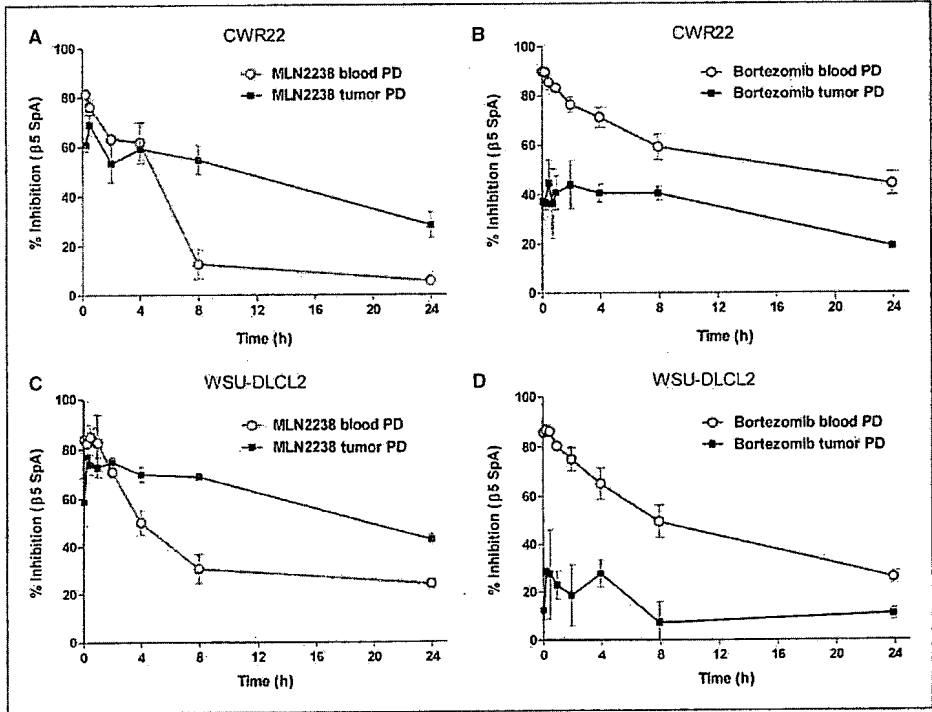


Figure 2. Blood and tumor proteasome inhibition versus time profile of MLN2238 (14 mg/kg; A and C) and bortezomib (0.8 mg/kg; B and D) following acute i.v. administration in CWR22 (A and B) and WSU-DLCL2 (C and D) tumor-bearing mice. Pharmacodynamic responses in blood and tumor were determined by measuring 20S proteasome $\beta 5$ enzyme inhibition in blood and tumor at different time points.

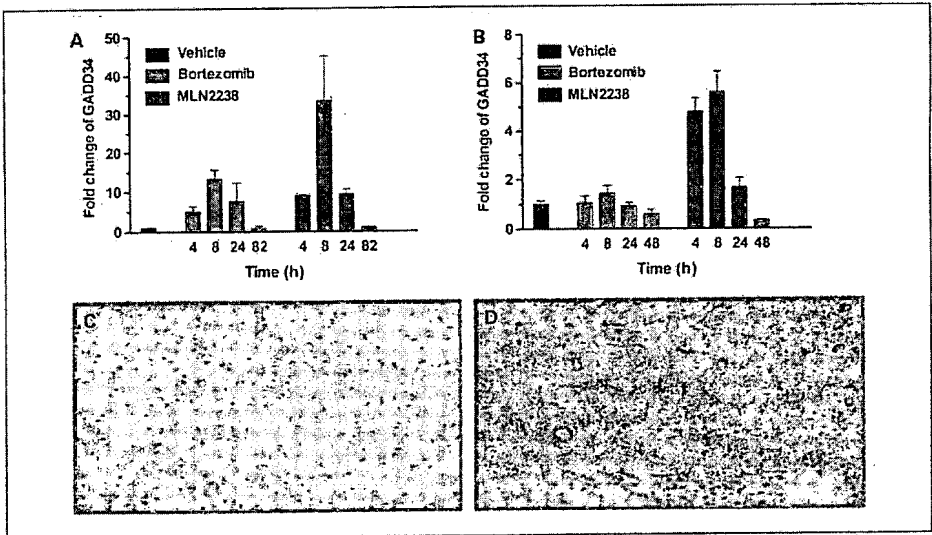


Figure 3. Pharmacodynamic responses in tumor were determined by measuring GADD34 protein levels at different time points via Western blot and quantitated with the Odyssey Infrared Imaging System. A and B, normalized GADD34 response versus time profile shown as fold change from vehicle control following acute i.v. administration of MLN2238 at 10 mg/kg and bortezomib at 0.8 mg/kg in CWR22 (A) and WSU-DLCL2 (B) tumor-bearing mice. Columns, mean of three tumors per group, except CWR22 vehicle group with four tumors and WSU-DLCL2 vehicle group with five tumors; bars, SD. Immunohistochemical staining (C and D) for GADD34 in CWR22 xenograft tumors 8 h following an acute i.v. dose of either vehicle (C) or MLN2238 at 14 mg/kg (D).

tial distribution phase and a long terminal $t_{1/2}$ (>24 hours; Fig. 1B). Due to extensive RBC partitioning, whole-body tissue distribution is most accurately reflected in blood volume distribution at steady state ($V_{dss, b}$) rather than

plasma volume distribution at steady state ($V_{dss, p}$). MLN2238 showed larger $V_{dss, b}$ (20.2 L/kg) compared with bortezomib (4.3 L/kg), providing supportive evidence that MLN2238 more easily moves from the blood compartment

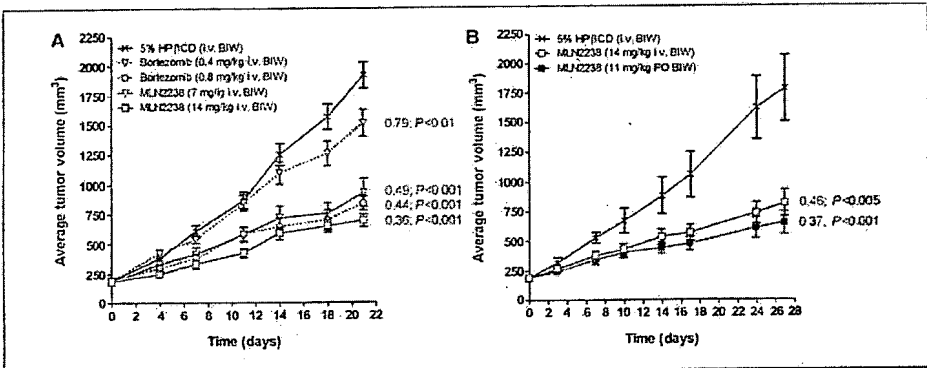


Figure 4. Antitumor activity of MLN2238 and bortezomib in CWR22 tumor-bearing mice ($n = 10$). A, animals were dosed i.v. twice weekly (BIW) with vehicle (5% HPβCD), bortezomib (0.4 and 0.8 mg/kg in 0.9% saline), and MLN2238 (7 and 14 mg/kg in 5% HPβCD). B, animals were dosed i.v. twice weekly with vehicle, i.v. twice weekly with MLN2238 (14 mg/kg), or orally twice weekly with MLN2238 (11 mg/kg). Points, average tumor volume in each treatment group; bars, SE. T/C and P values were calculated as described in Materials and Methods. A P value of ≤ 0.05 was considered significant.

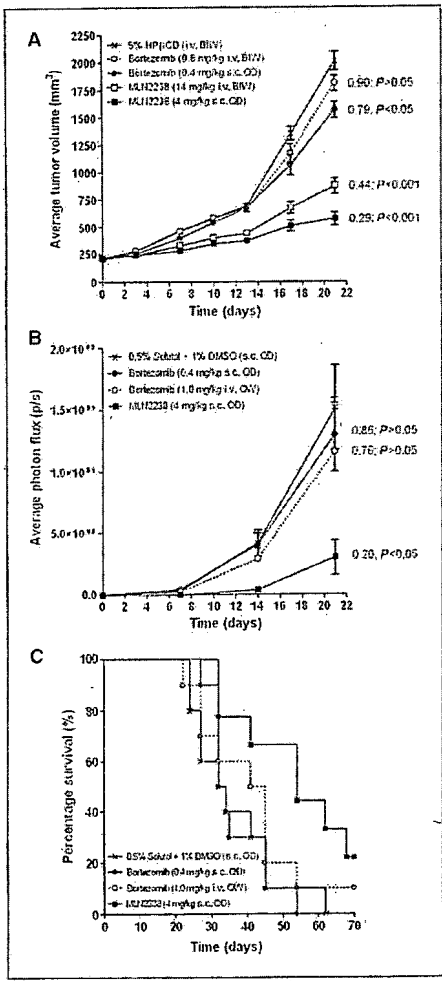


Figure 5. Antitumor activity of MLN2238 and bortezomib in two lymphoma xenograft models. Each treatment group consisted of 10 mice. **A**, antitumor activity in WSU-DLCL2 xenografts. Animals were dosed with vehicle (5% HPβCD i.v. twice weekly), bortezomib (0.8 mg/kg i.v. twice weekly or 0.4 mg/kg s.c. QD), or MLN2238 (14 mg/kg i.v. twice weekly or 4 mg/kg s.c. QD) for 3 consecutive weeks. **B**, antitumor activity in the OCI-Ly7-Luc disseminated lymphoma model. Animals were dosed with vehicle (0.5% Solulol + 1% DMSO s.c. QD), bortezomib (0.4 mg/kg in 0.9% saline s.c. QD), bortezomib (1.0 mg/kg in 0.9% saline i.v. QW), or MLN2238 (4 mg/kg in 0.5% Solulol + 1% DMSO s.c. QD) for 3 consecutive weeks. Points, average tumor volume; bars, SE. T/C and P values were calculated as described in Materials and Methods. A P value of ≤ 0.05 was considered significant. **C**, Kaplan-Meier survival profile.

into the tissue compartment. MLN2238 also showed moderate oral bioavailability (Table 1; Fig. 1C). To determine the pharmacokinetic profile of MLN2238 and bortezomib in a second species, Sprague-Dawley rats were administered a single i.v. dose of MLN2238 at either 0.3 or 0.2 mg/kg or bortezomib at 0.2 mg/kg. Both MLN2238 doses provided a greater plasma exposure (AUC_{0-48h} of 704 and 1,070 h·ng/mL for 0.2 and 0.3 mg/kg doses, respectively) compared with bortezomib (AUC_{0-48h} of 206 h·ng/mL), confirming that MLN2238 also has improved plasma exposure compared with bortezomib in rodents (Fig. 1D).

MLN2238 induces a greater pharmacodynamic response than bortezomib in xenograft tumors. To further evaluate the activity of MLN2238 *in vivo*, a series of pharmacodynamic studies were performed in CB17-SCID mice bearing human prostate (CWR22) or human lymphoma tumors (WSU-DLCL2). Pharmacodynamic responses were assessed by measuring (a) the degree of 20S proteasome inhibition and (b) the expression levels of the GADD34 protein.

Blood and tumor 20S proteasome inhibition versus time profiles were generated for MLN2238 and bortezomib from both CWR22 and WSU-DLCL2 xenografts (Fig. 2). The AUE was calculated from 0 to 24 hours (AUE_{0-24h}) for both blood and tumor (Table 1). These AUEs represent the summation of the pharmacodynamic effect over a defined period of time in a particular tissue compartment. Calculating the tumor to blood AUE ratio provided a functional reflection of the distribution and durable pharmacodynamic effect of the drug in different tissue compartments. The maximum level of blood proteasome inhibition (E_{max}) following an acute i.v. dose of either MLN2238 (83.1%) or bortezomib (88.3%) was nearly identical (Table 1). However, the duration of the effect differed between the two molecules, with bortezomib having a more sustained response and, therefore, a greater blood AUE than MLN2238 (Fig. 2B and D). In contrast to blood, MLN2238 showed both greater maximum and sustained tumor proteasome inhibition compared with bortezomib in both xenograft models (Table 1; Fig. 2A and C). The tumor to blood AUE ratio for MLN2238 in CWR22s and WSU-DLCL2s was 1.56 and 2.03, respectively, compared with 0.69 and 0.26 for bortezomib (Table 1). Consistent with the pharmacokinetic profiles described for these two molecules (i.e., MLN2238 has a greater Vd_{0-4h}), these results showed that MLN2238 had a greater pharmacodynamic effect in tumor compared with blood, whereas the opposite was true for bortezomib. Consistent with the improved tumor E_{max} in MLN2238-treated mice, these data confirm that MLN2238 had a greater overall tumor pharmacodynamic effect than bortezomib as assessed by 20S inhibition.

Additional pharmacodynamic markers were examined to study the downstream effects of proteasome inhibition. One of the consequences of proteasome inhibition is the accumulation of proteins associated with the endoplasmic reticulum (ER) stress pathway and the unfolded protein response (UPR) pathway (11, 45–50). One of these proteins is GADD34, a stress-inducible gene that is also upregulated in response to DNA damage, hypoxia, and energy depletion

(51). Western blot analyses were performed on tumors isolated from CWR22 and WSU-DLCL2 xenograft-bearing mice treated with either MLN2238 or bortezomib (Fig. 3). Increased GADD34 expression was seen in CWR22 xenograft tumors following MLN2238 or bortezomib treatment, whereas an even greater response was seen following MLN2238 treatment (Fig. 3A). Similarly, bortezomib treatment only led to a minor increase in GADD34 levels in WSU-DLCL2 xenograft tumors, whereas MLN2238 strongly induced its expression (Fig. 3B). To confirm these results, and to get a better understanding of the magnitude of response across individual cells within the tumor, immunohistochemical staining was performed using the anti-GADD34 antibody (Proteintech). Strong GADD34 staining, reflecting increases in GADD34 protein levels, was seen across the majority of tumor cells in CWR22 xenografts 8 hours after a single i.v. dose of MLN2238 at 14 mg/kg (Fig. 3D) compared with very low staining in vehicle-treated CWR22 xenografts (Fig. 3C). Approximately a 5-fold increase in the total number of GADD34-positive cells was seen at 8 and 24 hours following MLN2238 treatment compared with vehicle control-treated tumors (Supplementary Fig. S1). In WSU-DLCL2 xenograft tumors, bortezomib treatment led to only a minor increase in GADD34 levels measured by Western blot, whereas MLN2238 strongly induced its expression (Fig. 3B). In addition, examining levels of ATF3, another gene upregulated during ER stress and UPR activation (48, 52, 53), revealed a similar pattern, with a greater number of cells staining positively for ATF3 following treatment of WSU-DLCL2 xenograft tumors with MLN2238 compared with bortezomib (Supplementary Fig. S2). These results confirm that the improved tumor exposure seen with MLN2238 translated into an improved tumor pharmacodynamic response both at the level of and downstream from the proteasome.

MLN2238 shows antitumor activity in the CWR22 xenograft model. To confirm that the pharmacodynamic responses seen in CWR22 xenografts would translate into antitumor activity, a series of efficacy experiments were performed comparing MLN2238 with bortezomib.

The antitumor effects of MLN2238 dosed at 14 mg/kg i.v. or 7 mg/kg i.v. were compared with bortezomib dosed at 0.8 mg/kg i.v. or 0.4 mg/kg i.v. on a twice weekly regimen (Fig. 4A). The high dose for both MLN2238 and bortezomib showed similar antitumor activity in this model ($T/C = 0.36$ and 0.44 , respectively). However, MLN2238 (7 mg/kg) showed greater efficacy at a 0.5 MTD dose compared with a 0.5 MTD dose of bortezomib (0.4 mg/kg; $T/C = 0.49$ compared with $T/C = 0.79$, respectively; Fig. 4A).

MLN2238 has moderate orally bioavailability (Table 1). In Fig. 4B, we show that oral dosing of MLN2238 resulted in antitumor activity in the CWR22 xenograft model ($T/C = 0.37$). Taken together, these results show that the human prostate CWR22 model is responsive to proteasome inhibition. Furthermore, a direct comparison between MLN2238 and bortezomib revealed similar antitumor activity when dosed at their respective MTDs; however, when both compounds were dosed at their respective 0.5 MTDs, MLN2238 showed improved activity over bortezomib (Fig. 4A).

MLN2238 shows improved efficacy compared with bortezomib in two models of lymphoma. MLN2238 showed greater tumor pharmacodynamic responses in WSU-DLCL2 xenografts compared with bortezomib (Table 1; Figs. 2 and 3). To assess whether the more robust pharmacodynamic response translated to greater antitumor activity, an efficacy study was performed in WSU-DLCL2 tumor-bearing mice. The antitumor effects of MLN2238 [dosed at 14 mg/kg i.v. twice weekly or 4 mg/kg s.c. once daily (QD)] were directly compared with bortezomib (dosed at 0.8 mg/kg i.v. twice weekly or 0.4 mg/kg s.c. QD; Fig. 5A). In this experiment, neither of the bortezomib doses showed strong antitumor activity ($T/C = 0.79$ and 0.9 for 0.8 mg/kg i.v. and 0.4 mg/kg s.c., respectively). In contrast, both intermittent and continuous MLN2238 dosing regimens showed strong antitumor activity ($T/C = 0.44$ and 0.29 for 14 mg/kg i.v. and 4 mg/kg s.c., respectively) and generated a greater apoptotic response in tumor tissue as measured by levels of cleaved caspase-3 (Supplementary Fig. S3).

The antitumor activity of MLN2238 and bortezomib was evaluated in a disseminated model of lymphoma. The ability of both drugs to reduce tumor burden and improve overall survival was assessed in this systemic lymphoma model. NOD-SCID mice were inoculated with OCI-Ly7-Luc cells expressing a luciferase reporter gene. Bioluminescent scans, obtained via quantitative Xenogen imaging, allowed tumor growth to be tracked over time in live animals. The strongest antitumor response was seen following treatment with MLN2238 at 4.0 mg/kg s.c. QD ($T/C = 0.20$; Fig. 5B). This dosing regimen also significantly prolonged overall survival in this model compared with vehicle-treated controls (median survival was 54 versus 33 days, $P = 0.05$; Fig. 5C). Much weaker antitumor responses were seen following bortezomib treatment at 0.4 mg/kg s.c. QD or 1.0 mg/kg i.v. once weekly ($T/C = 0.86$ and 0.76 , respectively). These bortezomib dosing regimens also did not significantly prolong survival (median survival was 33 and 43 days, respectively; $P > 0.99$ for both; Fig. 5C).

In summary, we have identified a second-generation small-molecule inhibitor of the proteasome. It has different physicochemical properties compared with bortezomib, including a shorter proteasome dissociation $t_{1/2}$, which we believe plays a critical role in the ability of this molecule to distribute into tissues. Improved pharmacokinetic and tolerability allow this molecule to be administered at higher doses, resulting in greater blood and plasma exposures. Consistent with these findings, we found a greater pharmacodynamic response in multiple xenograft models treated with MLN2238 compared with bortezomib, particularly in tumor, supporting our hypothesis that MLN2238 has improved distribution characteristics. Superior bioavailability also allows this molecule to be dosed orally, whereas bortezomib is restricted to i.v. and s.c. dosing regimens to achieve acceptable exposure levels. Data generated from both s.c. and disseminated xenograft efficacy studies show that MLN2238 has greater antitumor activity when administered by either intermittent or continuous dosing regimens and improves overall survival compared with bortezomib. Taken together, these data support the clinical

development of MLN9708 for both hematologic and solid tumor indications.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Correction

**Correction: Evaluation of the Proteasome
Inhibitor MLN9708 in Preclinical Models of
Human Cancer**

In this article (Cancer Res 2010;70:1970-80), which was published in the March 1, 2010 issue of *Cancer Research* (1), Paul Hales (Millennium Pharmaceuticals, Inc., Cambridge, Massachusetts) should have been included as the ninth author. The online article has been changed to reflect this correction and no longer matches the print.

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Correction

**Correction: Online Publication Dates for
Cancer Research April 15, 2010 Articles**

The following articles in the April 15, 2010 issue of *Cancer Research* were published with an online publication date of April 6, 2010 listed, but were actually published online on April 13, 2010:

Garmy-Susini B, Avraamides CJ, Schmid MC, Foubert P, Ellies LG, Barnes L, Feral C, Papayannopoulou T, Lowy A, Blair SL, Cheresh D, Ginsberg M, Varner JA. Integrin $\alpha 4 \beta 1$ signaling is required for lymphangiogenesis and tumor metastasis. *Cancer Res* 2010;70:3042–51. Published OnlineFirst April 13, 2010. doi:10.1158/0008-5472.CAN-09-3761.

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Cancer Research

Evaluación del inhibidor de proteasoma MLN9708 en Modelos Preclínicos de cáncer humano.

Erik Kuppérman, Edmund C. Lee, Yueying Cao, et al.

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Terapéutica, metas y biología química.

Evaluación del inhibidor de proteasoma MLN9708 en Modelos Preclínicos de cáncer humano

Erik Kuppérman, Edmund C. Lee, Yueying Cao, Bret Bannerman, Michael Fitzgerald, Allison Berger, Jie Yu, Yu Yang, Paul Hales, Frank Bruzzese, Jane Liu, Jonathan Blank, Khristofer Garcia, Christopher Tsu, Larry Dick, Paul Fleming, Li Yu, Mark Manfredi, Mark Rolfe, y Joe Bolen.

Resumen

La proteasoma fue validada como un blanco de la oncología luego del éxito clínico de VELCADE (bortezomib) para inyección para el tratamiento de mieloma múltiple y linfoma de células de manto recurrente. En consecuencia, diversos grupos están detrás del desarrollo de inhibidores de proteasoma de moléculas pequeñas adicionales tanto para indicaciones hematológicas como de tumores sólidos. En el presente artículo describimos el MLN9708, un inhibidor de proteasoma selectivo, biodisponible oralmente, de segunda generación que se encuentra en la fase I de desarrollo clínico. MLN9708 tiene una vida media de disociación de proteasoma más corta, así como farmacocinética, farmacodinámica y actividad antitumor mejorada en comparación con el bortezomib. MLN9708 tiene una mayor distribución de volumen de sangre en estado estable, y los análisis de la inhibición de la proteasoma 20S, así como los marcadores de la respuesta de proteína desplegada confirmaron que MLN9708 tiene mayores efectos farmacodinámicos en los tejidos a comparación con el bortezomib. MLN9708 mostró actividad tanto en tumores sólidos como en modelos de xenoinjertos hematológicos, y encontramos una correlación entre mayores respuestas farmacodinámicas y actividad antitumor mejorada. Por otro lado, la actividad antitumor fue evidenciada a través de múltiples rutas de dosificación, incluyendo sonda oral. En conjunto, esta información soporta el desarrollo clínico de MLN9708 tanto para indicaciones hematológicas y de tumores sólidos. *Cancer Res*; 70(5); 1970-80. ©2010 AACR.