



No. 10-063351 - 00005-0000

Fecha: 2011-12-21 16:41:12 Dep. 2020 DIR.NUEVASCR
Tra. 11 PATENTEIYII Eve: 378 FASENACIONALI
Act. 449 RESPUESOPOSICI Folios: 21

Señores

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

DIVISION DE NUEVAS CREACIONES

E. _____ S. _____ D. _____

Re: Expediente N° 10-063.351

Solicitud de Patente de Invención Titulada: "BIFOSFONATOS DE ALQUILO DE 2 A 5 ÁTOMOS DE CARBONO-IMIDAZOL".

Solicitante: NOVARTIS AG

Nuestra Ref.: P2010/000039

RESPUESTA A LA OPOSICIÓN NOTIFICADA MEDIANTE EL OFICIO No. 15001
POR FIJACIÓN EN LISTA No. 358, DE FECHA 23 DE SEPTIEMBRE DE 2011

CARLOS R. OLARTE, mayor de edad y vecino de Bogotá, D.C., identificado tal como aparece al pie de mi firma, en mi carácter de apoderado de la sociedad solicitante, me permito manifestar lo siguiente, dentro del plazo legal previsto en el artículo 43 de la Decisión 486, ante la oposición interpuesta por el doctor José Luis Salazar Villamizar, actuando como apoderado de la sociedad LAFRANCOL S.A.

1. En primer lugar, con el ánimo de brindar mayor claridad a la materia reivindicada, me permito presentar un **Capítulo Reivindicatorio Modificado**, que reemplaza en su totalidad al Capítulo Reivindicatorio presentado el día 16 de Septiembre de 2010, del cual se han eliminado las Reivindicaciones 6 y 7 y donde las reivindicaciones restantes se han reenumerado.

Valga anotar que las modificaciones efectuadas al Capítulo Reivindicatorio cumplen a cabalidad con lo estipulado en el Artículo 34 de la Decisión 486, ya que éstas de ninguna manera implican una ampliación de la protección correspondiente a la divulgación contenida en la solicitud inicial, por el contrario, éstas corresponden a una franca limitación de la materia reivindicada.

2. a- El Opositor señala que los métodos de tratamiento no son materia patentable, de acuerdo con lo establecido en el artículo 20(d) de la Decisión 486.

b- Considero superada la anterior objeción, en tanto que las Reivindicaciones 6 y 7 han sido eliminadas del Capítulo Reivindicatorio Modificado adjunto.

3. a- El Opositor argumenta que la presente solicitud carece de nivel inventivo a la luz de lo revelado en Wilder et al¹ (D1), Kotsikorou et al² (D2), Xie et al³ (D3), WO2002/043738 (D4) y Fernández et al⁴ (D5).

El Opositor simplemente argumenta que los documentos D1 a D4 describen compuestos relacionados con los compuestos de la presente invención, enseñando que tales bifosfonatos son muy versátiles ya que mantienen su actividad a pesar de su modificación estructural. El Opositor también argumenta que las reacciones con oxihaluros de fósforo para obtener bifosfonatos fueron previamente enseñadas en D5.

b- i) En primer lugar, me permito señalar lo siguiente respecto al arte previo citado por el Opositor:

- En relación con D1, este documento enseña que los bifosfonatos de mayor potencia corresponden al tipo que contiene a una fracción heteroaromática con al menos un átomo de nitrógeno, unidos únicamente a través de un grupo metileno a la unidad germinal de bifosfonato, donde el miembro más importante de ese género es el ácido zoledrónico (compuesto **6i** en la tabla 8 de D1, que reproduzco a continuación). Dicho ácido zoledrónico es el único que en un ensayo con ratas con las glándulas tiroideas y paratiroides ectomizadas⁵, muestra de lejos, la mejor ED₅₀ de 0.07µg/Kg cuando se compara con los compuestos de imidazolilo no-, mono- o di-metilados.

¹ Leo Wilder *, Knut A. Jaeggi, Markus Glatt, Klaus Muller, Rolf Bachmann, Michael Bisping, Ruth Anne Born, Reto Cortesi, Gabriela Guiglia, Heidi Jeker, Remy Klein, Ueli Ramseier, Johann Schmid, Gerard Schreiber, Yves Seltenmeyer and Jonathan R. J. Green. Highly Potent Geminal Bisphosphonates. From Pamidronate Disodium (Aredia) to Zoledronic Acid (Zometa). Med Chem 2002, 45 (17), pp 3721-3738.

² Evangelia Kotsikorou and Eric Oldfield. A quantitative structure-Activity Relationship and pharmacophore modeling Investigation of Aryl-X and heterocyclic bone resorption as agents bisphosphonates. Medicinal Journal of Chemistry, 2003, Vol. 46, No. 14.

³ A. Xie, C. Liao, Z. Ning, W. Hu, X. Lu, L. Shi and J. Zhou. Quantitative Structure-Relationship Activity study of bisphosphonates. Internet Electron.K. Mol. Des. 2004, 3, 622-650, <http://www.biochempress.com>.

⁴ Christian Fernandez, Rodrigo Souza Leite and Fernando Mauro Lanka. Disphosphonates: Síntese, Análises Químicas E Aplicações Farmacológicas. Quim. Nova, Vol 28, No. 2.274-280, 2005.

⁵ Green et al., J. Bone Miner. Res, 1994, 9(5), 745-51):

Compuesto	Estructura	ED ₅₀ [μg/kg]
6a		5
6b		0.6
6c		25
6d		0.3
6f		15
6h		1.5
6i		<u>0.07</u>
6j		45
6k		3
6l		1.5

De los anteriores compuestos ilustrados en D1 resulta claro que nada en esta referencia enseña que la fracción heteroaromática de la presente invención podría sustituirse en la posición 4 o 5 por un grupo alquilo con al menos dos átomos de carbono, preservando su actividad como inhibidor de la excesiva e inadecuada resorción ósea.

- En cuanto a D2 y D3, éstos describen resultados de relación estructura-actividad para series de bifosfonatos heterobíciclicos, incluyendo algunos de los compuestos de D1, así como el compuesto 35 de D2 y el Compuesto 68 de D3 (que corresponde al compuesto 61 de D1), los cuales comprenden un grupo 4,5-dimetil-imidazol-1-il. De la misma manera, D4 en el mejor de los casos menciona bifosfonatos que comprenden grupos 1-metil-imidazo-2-il, 1-benzil-imidazo-2-il y 2-metil-imidazo-5-il. En conclusión, ninguna de las enseñanzas de D2, D3 y/o D4, sugiere incluir un grupo alquilo con al menos dos átomos de carbono en las posiciones 4 o 5 del anillo imidazol-1-il.

En particular, resulta interesante observar que en D3 los compuestos 63 y 64, cuando se comparan con el compuesto 61 con dos átomos de hidrógeno en las posiciones R^1 y R^2 en los carbonos del anillo en un experimento *in vivo*, muestran un ED_{50} menos preferible por un factor de 50 a 5 respectivamente.

Asimismo, los compuestos 65, 67 y 68, comparables a los compuestos de la presente invención en donde el anillo está también enlazado por un nitrógeno, muestran que hay un incremento en la actividad de aproximadamente 43 o 21 veces (para los compuestos 67 y 68 respectivamente) cuando no se incluye un sustituyente metilo, esto es, cuando R^1 , R^2 y R^3 son hidrógeno.

D2 también muestra que el compuesto 40 (6k) allí mencionado (el cual está sustituido por metilo) tiene una EC_{50} significativamente mayor cuando se compara con el compuesto 30 (6i) (el cual no está sustituido) y eso sugiere que la sustitución disminuiría la actividad y coincidiría con los datos proporcionados en D3.

- Respecto a D5, me permito señalar, que este documento enseña la síntesis de bifosfonatos ya sea por reacción entre un anhídrido acético con ácido fosfórico, por reacción de un ácido carboxílico y ácido fosfórico en presencia de tricloruro de fósforo, por reacción de un cloruro de acilo en presencia de tris(trimetilsilil)fosfato y metanólisis, y por reacciones de bishidrofosforilación de alquinos terminales. Sin embargo, nada en D5 enseña o sugiere que la preparación de los compuestos de la presente

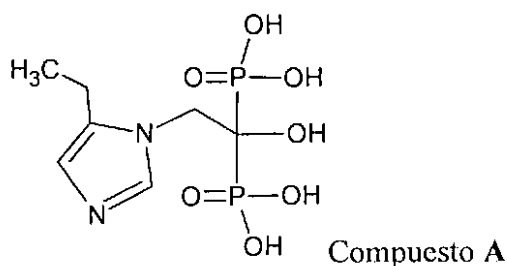
invención puedan ser formados por reacción de un ácido carboxílico sustituido en el imidazol con un oxi-haluro de fósforo.

ii) Para resumir, una persona medianamente versada en la materia en la búsqueda de compuestos más eficientes y no solamente compuestos alternativos, llegaría necesariamente a la conclusión de que los compuestos de tipo imidazol, unidos por medio de un N deben ser preferiblemente no sustituidos. Por lo tanto la persona medianamente versada en la materia no estaría motivada a adicionar ninguna cadena carbonada sobre el anillo del imidazol. Es más, el arte previo proporciona enseñanzas contrarias que no conducirían específicamente a compuestos sustituidos con etilo sobre el grupo imidazol. En conclusión, es contra intuitivo para cualquier persona medianamente versada en la materia adicionar sustituyentes sobre el anillo imidazol-1-il de la presente invención, y mucho menos producirlos con el sustituyente etilo específico.

Adicionalmente, al analizar los compuestos de la invención sobre la Farnesyl pirofosfato sintetasa (FPPS), éstos mostraron sorprendentemente buena inhibición en comparación con compuestos cercanos del arte previo.

iii) Finalmente, me permito presentar datos comparativos específicos que claramente evidencian que los compuestos reivindicados cumplen con el requisito de nivel inventivo y no solo son evidentemente derivados de lo revelado en el arte previo.

En un ensayo realizado usando ratas deficientes de estrógeno, para la determinación de la prevención de la pérdida de masa ósea después de la ovariectomía en ratas hembra de 7 meses de edad (ensayo OVX), se comparó la duración de la acción de una inyección intravenosa única 4µg/kg de un nuevo aminobifosfonato –compuesto A (el compuesto de la Reivindicación 5 de la presente invención) frente a la misma dosis de ácido zoledrónico (Compuesto 6i de D1).

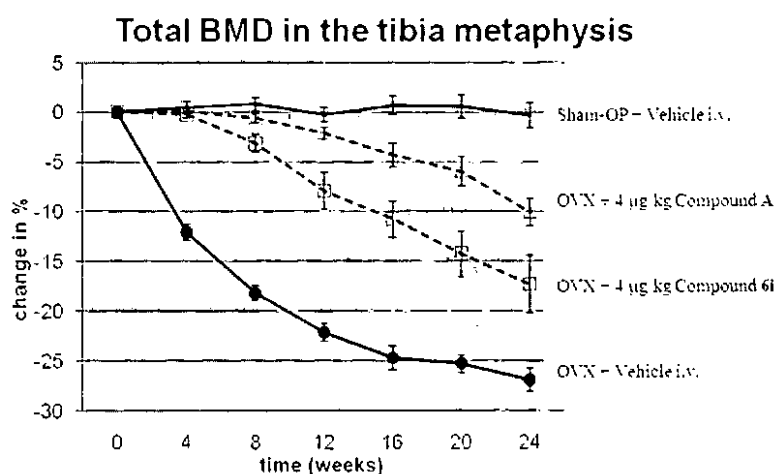


Los dos bifosfonatos se administraron al inicio del estudio (semana 0), y se monitoreó el cambio en la densidad mineral de los huesos (BMD) en la metáfisis proximal de la tibia a intervalos mensuales durante 6 meses. Se analizó el total de la sección transversal (BMD total: hueso

cortical y esponjoso), un análisis separado de BMD en hueso esponjoso, y el espesor medio de la corteza cortical.

BMD total: En animales con falsa cirugía, la BMD total se mantuvo estable durante los seis meses del periodo de monitoreo. Por el contrario las ratas deficientes de estrógeno OVX mostraron una rápida disminución en la BMD total entre las primeras 12 semanas que se mantuvo estable a -25% para el resto del periodo de observación.

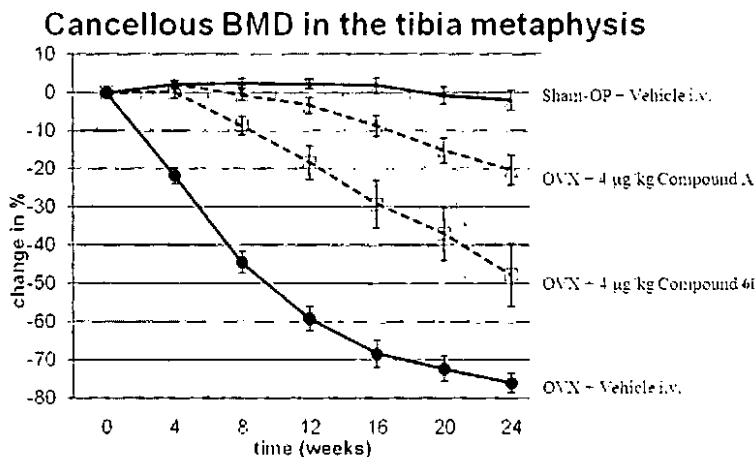
Una dosis única i.v. del compuesto **6i** protegió completamente las ratas OVX contra la caída inducida en la BMD total por 4 semanas, mientras que la misma dosis del compuesto **A** protegió completamente por 12 semanas. Posteriormente, la BMD total comenzó a caer, lo que indica el inicio de la pérdida de la masa luego de 4 semanas (Compuesto **6i**) y 12 semanas (Compuesto **A**) respectivamente. Con ambos compuestos, se observó un persistente efecto anti-resorcivo durante el periodo de observación de 6 meses, sin embargo, el compuesto **A** se desempeñó significativamente mejor que el compuesto **6i** en todos los tiempos (Fig 1):



BMD en hueso esponjoso: En animales con falsa cirugía, la BMD en hueso esponjoso se mantuvo estable sobre los seis meses de monitoreo. Por el contrario, en las ratas deficientes de estrógeno se observó una rápida disminución en la BMD en hueso esponjoso entre las primeras 16 semanas con pérdida de masa ósea disminuyendo después de llegar al -75% a las 24 semanas.

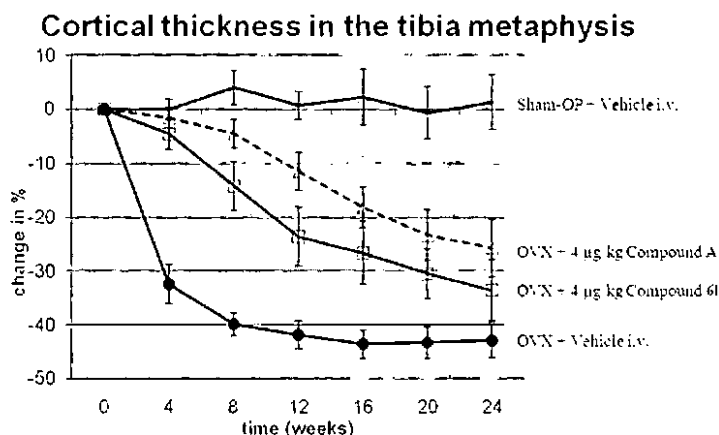
Una dosis única i.v. del compuesto **6i** protegió completamente las ratas OVX contra la caída inducida en la BMD de hueso esponjoso por 4 semanas, mientras que la misma dosis del compuesto **A** protegió completamente por 12 semanas. Posteriormente, la BMD en hueso esponjoso comenzó a caer, lo que indica el inicio de la pérdida de la masa luego de 4 semanas (Compuesto **6i**) y 12 semanas (Compuesto **A**) respectivamente. Con ambos compuestos, se observó un persistente efecto anti-resorcivo durante el periodo de observación de 6 meses, sin

embargo, el compuesto A se desempeñó significativamente mejor que el compuesto 6i en todos los tiempos (Fig 2):



Espesor Cortical: En animales con falsa cirugía, el Espesor Cortical se mantuvo estable sobre los seis meses de monitoreo. Por el contrario, en las ratas deficientes de estrógeno se observó una rápida disminución en el Espesor Cortical entre las primeras 8 semanas con pérdida de masa ósea disminuyendo después de llegar al -45% a las 16 semanas.

Una dosis única i.v. del compuesto 6i protegió completamente las ratas OVX contra la caída inducida en el Espesor Cortical por 4 semanas, mientras que la misma dosis del compuesto A protegió completamente por 8 semanas. El Espesor Cortical comenzó a caer, lo que indica el inicio de la pérdida de la masa luego de 4 semanas (Compuesto 6i) y 8 semanas (Compuesto A) respectivamente. Con ambos compuestos, se observó un persistente efecto anti-resorciivo durante el periodo de observación de 6 meses, sin embargo, el compuesto A se desempeñó significativamente mejor que el compuesto 6i en todos los tiempos (Fig 3):



En conclusión, una dosis única i.v. de 4µg/kg del compuesto A fue totalmente protectora para los huesos en 12 semanas en todos los parámetros que fueron monitoreados. En comparación, el compuesto 6i, con una dosis única de 4µg/kg, proporcionó un efecto protector solamente por 4

semanas, con una pérdida ósea significativa posterior, de 8 a 12 semanas. Esto indica que el compuesto A puede ser hasta 5 veces más potente que el medicamento de bifosfonato basado especialmente en el compuesto 6i, el cual es comercializado hoy bajo el nombre de Aclasta/Reclast, como una dosis única de infusión anual de 5mg por paciente para el tratamiento de osteoporosis post-menopáusica.

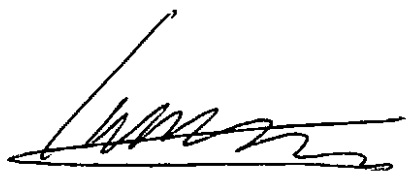
Adicionalmente, basados en los datos disponibles para alendronato en la publicación Gasser et al,⁶ se puede estimar que el compuesto A es aproximadamente 25 veces más potente que el de uso más frecuente por vía oral, el bifosfonato Fosamax (alendronato), el cual se encuentra en el mercado para el tratamiento de la osteoporosis post-menopáusica.

Estos estudios en ratas también sugieren que la dosis efectiva anual del compuesto A en humanos podría potencialmente ser tan baja como 1 mg/año, con la condición de que no haya diferencias entre las especies.

En general, los resultados proporcionan evidencia preclínica de la eficacia y la extraordinaria potencia del aminobifosfonato antirresortivo, compuesto A.

4. Por todo lo anterior, considero que las objeciones presentadas por el Opositor han sido resueltas, y por consiguiente solicito respetuosamente que el Despacho proceda con el examen de fondo de la presente solicitud.

Atentamente,



CARLOS R. OLARTE
C. C. No. 79.782.747 de Bogotá
T. P. 74.295

Anexos:

- Capítulo Reivindicatorio Modificado.
- Gasser JA, Ingold P, Venturiere A, Shen V, and Green JR (2008) Long-Term Protective Effects of Zoledronic Acid on Cancellous and Cortical Bone in the Ovariectomized Rat. J Bone Miner Res; 23:544-51.

⁶ Gasser JA, Ingold P, Venturiere A, Shen V, and Green JR (2008) Long-Term Protective Effects of Zoledronic Acid on Cancellous and Cortical Bone in the Ovariectomized Rat. J Bone Miner Res; 23:544-51

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 900176089-2

CERTIFICACION DE EXCEDENTE : 11 - 88,283
FECHA : SEPTIEMBRE 8 DE 2011

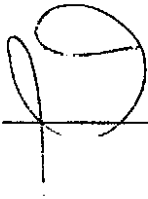
***** CONSIGNACION *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PGO	Vr. PAGO
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OLARTE RAISBEK	RECIBO DE CAJA			32498	07/04/2011	432,000.00
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OLARTE RAISBEK	RECIBO DE CAJA			38097	11/05/2010	114,000.00
OLARTE RAISBEK	RECIBO DE CAJA			38000	13/05/2010	52,000.00
...MAS INFORMACION NO IMP			TOTAL			2,920,000.00

***** CONCEPTO *****

CANT.	RENTISTICO	CONCEPTO	TOTAL CONCEPTO
1	50005-01-09	OTROS SERVICIOS DE PROPIEDAD I	
		99 OTROS SERVICIOS ADMINISTRATIVOS	5,000.00
		TOTAL :	\$ 5,000.00

SON: CINCO MIL PESOS

RESPONSABLE : 

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

No. 10-063351- -00005-0000
Fecha: 2011-12-21 16:41:12 Dep. 2020 DIR.NUEVASCR
Tra. 11 PATENTEIYII Eve: 378 FASENACIONALI
Act. 449 RESPUESOPOSICI Folios: 21

RECIBO DE CAJA APLICADO AL EXPEDIENTE No. _____

ESTA CERTIFICACION DE EXCEDENTE DE PAGO SOLO PODRA SER UTILIZADA
COMO PARTE DE LA CANCELACION DE UNA TARIFA VIGENTE Y NO PODRA SER
SUSTITUIDA POR NINGUN RECIBO.

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
NIT : 800.176.089-2
- / -

Industria y Comercio
SUPERINTENDENCIA

RECIBO DE CAJA

No. 11 - 0093595

Bogotá D.C., Septiembre 23 de 2011 - 10:49:34

RECIBIDO DE : OLARTE MOURE & ASOCIADOS

NI 830.122.870

*** Soporte del Pago ***

TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PAGO	VR. PAGO
RECIBO DE CA	999999999	88392	20/10/2010		114.000.00

*** Conceptos Pagados ***

CANT.	RENTISTICO	CONCEPTO	Vr.UNDITARIO	Vr.CONCEPTO
1	50005-01-09 OTROS SERVICIOS DE PROPIEDAD INDUSTRIAL	99 OTROS SERVICIOS ADMINISTRATIVOS	114.000.00	114.000.00
				\$114.000.00

SON: **CIENTOCATORCE MIL PESOS MONEDA CORRIENTE**

Responsable:

Recibo de Caja Aplicado al Expediente No.

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 10-063351- -00005-0000

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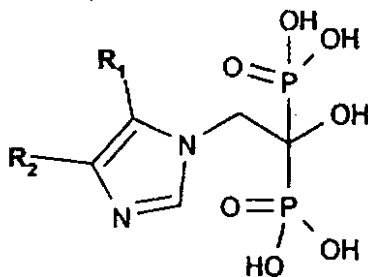
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Septiembre 23 de 2011 - 10:49:34

CAPÍTULO REIVINDICATORIO MODIFICADO (Modificaciones Voluntarias)

1. Un compuesto de la Fórmula I:



(I)

en donde uno de R₁ y R₂ es hidrógeno, y el otro es alquilo de 2 a 5 átomos de carbono que puede estar ramificado o no ramificado, o un éster, y/o una sal del mismo.

2. Un compuesto de la Fórmula I, de acuerdo con la Reivindicación 1, en donde:

R₁ es alquilo de 2 a 5 átomos de carbono, en especial propilo, isopropilo, butilo normal, butilo secundario, butilo terciario, isobutilo, o especialmente etilo, y

R₂ es hidrógeno,

o un éster del mismo, y/o una sal (en especial farmacéuticamente aceptable) del mismo.

3. Un compuesto de la Fórmula I, de acuerdo con la Reivindicación 1, en donde:

R₁ es hidrógeno, y

R₂ es alquilo de 2 a 5 átomos de carbono, en especial propilo, isopropilo, butilo normal, butilo secundario, butilo terciario, isobutilo, o especialmente etilo,

o un éster del mismo, y/o una sal (en especial farmacéuticamente aceptable) del mismo.

4. Un compuesto de la Fórmula I, de acuerdo con la Reivindicación 1, en donde R₁ es hidrógeno, y

R₂ es etilo,

o un éster del mismo, y/o una sal (en especial farmacéuticamente aceptable) del mismo.

5. Un compuesto de la Fórmula I, de acuerdo con la Reivindicación 1, en donde:

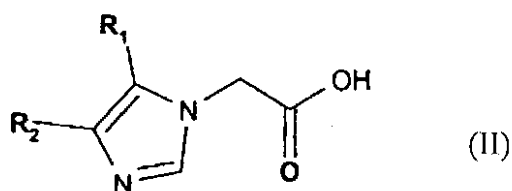
R₁ es etilo, y

R₂ es hidrógeno, o un éster del mismo, y/o una sal (en especial farmacéuticamente aceptable) del mismo.

6. Una composición farmacéutica, la cual comprende un compuesto de la Fórmula I, un éster, y/o una sal farmacéuticamente aceptable del mismo, de acuerdo con cualquiera de las Reivindicaciones 1 a 5, y cuando menos un vehículo farmacéuticamente aceptable.

7. Un proceso o método para la fabricación de un compuesto de la Fórmula I, un éster, y/o una sal del mismo, de acuerdo con cualquiera de las Reivindicaciones 1 a 5, el cual comprende:

hacer reaccionar un compuesto de ácido carboxílico de la Fórmula II:



en donde R_1 y R_2 son como se definen para un compuesto de la Fórmula I en cualquiera de las reivindicaciones 1 a 5, con oxi-haluro de fósforo, para dar un compuesto de la Fórmula I, o una sal del mismo,

y si se desca, convertir un compuesto libre que se pueda obtener de la Fórmula I en su sal, convertir una sal que se pueda obtener de un compuesto de la Fórmula I en el compuesto libre, y/o convertir una sal que se pueda obtener de un compuesto de la Fórmula I en una sal diferente del mismo.

Long-Term Protective Effects of Zoledronic Acid on Cancellous and Cortical Bone in the Ovariectomized Rat

Jürg A Gasser,¹ Peter Ingold,¹ Andrea Venturiere,¹ Victor Shen,² and Jonathan R Green¹

ABSTRACT: Current bisphosphonate therapies effectively prevent bone loss in postmenopausal women. We studied the effect of a single intravenous dose of ZOL in ovariectomized rats. Protection from bone loss was dose dependent, lasting for up to 32 weeks, supporting the rationale for an annual intravenous dosing regimen of ZOL for treatment of postmenopausal osteoporosis.

Introduction: Once-yearly dosing with zoledronic acid (ZOL) 5 mg can increase BMD and reduce fracture rate in postmenopausal women with low BMD. The primary objective of this study was to determine the duration of bone protective effects of a single dose of ZOL in ovariectomized rats, an animal model of postmenopausal osteopenia. Secondary objectives were to determine the effects on bone turnover and mechanical properties.

Materials and Methods: Female Wistar rats (10 per group) received single intravenous doses of ZOL 0.8, 4, 20, 100, or 500 $\mu\text{g/kg}$, alendronate 200 $\mu\text{g/kg}$, or isotonic saline 4 days before bilateral ovariectomy. Sham-operated controls were pretreated with saline. Mass and density of cancellous and cortical bone (pQCT) were measured at 4-wk intervals for 32 wk. Bone architecture (μCT), bone formation dynamics (fluorochrome label-based histomorphometry), and biomechanical strength in compression testing were also assessed at 32 wk.

Results: Ovariectomy-associated BMD loss was significantly attenuated for 32 wk by ZOL ≥ 4 $\mu\text{g/kg}$ for total BMD, ZOL ≥ 20 $\mu\text{g/kg}$ for cortical BMD, and ZOL ≥ 4 $\mu\text{g/kg}$ for cancellous BMD ($p < 0.01$ versus ovariectomized controls). Alendronate 200 $\mu\text{g/kg}$ was of equivalent potency to ZOL 20 $\mu\text{g/kg}$. Ovariectomy-associated decreases in trabecular architectural parameters were dose-dependently attenuated by ZOL. Alendronate 200 $\mu\text{g/kg}$ was equivalent to ZOL 20 $\mu\text{g/kg}$. The bone resorption marker TRACP5b indicated transient suppression of elevated osteoclast activity by ZOL relative to OVX-rats even at the lowest dose of 0.8 $\mu\text{g/kg}$, whereas at 100–500 $\mu\text{g/kg}$, the effect was significant relative to the OVX control for the entire duration of the study of 32 wk. Bone formation parameters were not significantly affected by ZOL 20 $\mu\text{g/kg}$ but were significantly reduced by ZOL 100–500 $\mu\text{g/kg}$. Alendronate 200 $\mu\text{g/kg}$ was equivalent to ZOL 100 $\mu\text{g/kg}$. ZOL produced dose-related improvements in bone strength parameters after ovariectomy. Alendronate 200 $\mu\text{g/kg}$ was of similar potency to ZOL 20 $\mu\text{g/kg}$.

Conclusions: The duration and magnitude of the bone-protecting effect of a single intravenous dose of ZOL in ovariectomized rats is dose dependent and lasts for up to 32 wk. Compared with alendronate, ZOL shows 10-fold higher potency in preventing bone loss. These data support the use of an annual intravenous ZOL dosing regimen for the treatment of osteoporosis.

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Key words: osteoporosis, bone loss, bisphosphonate, single dose, zoledronic acid

INTRODUCTION

ESTROGENS PLAY A critical role in maintaining normal bone mass. In postmenopausal women, estrogen deficiency results in clinically significant bone loss and increased fracture risk. Current bisphosphonate therapies are effective treatments for bone loss. Zoledronic acid (ZOL),

a new-generation bisphosphonate, is the most potent inhibitor of osteoclast-mediated bone resorption in *in vitro* assays.⁽¹⁾ Long-term administration of ZOL as weekly subcutaneous injections has also been shown to prevent bone loss and maintain mechanical strength in ovariectomized rats and rhesus monkeys.^(2–4) In patients with Paget's disease of bone, a single infusion of ZOL 5 mg produces a rapid and sustained normalization of serum alkaline phosphatase.⁽⁵⁾ Moreover, once yearly intravenous dosing with ZOL 5 mg increased BMD and markedly reduced fracture rate in postmenopausal women with low BMD,⁽⁶⁾ indicating a new

Dr Gasser is employed by Novartis. Drs Ingold, Venturiere, and Green are employed by Novartis and hold stock in Novartis. Dr Shen states that he has no conflicts of interest.

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paradigm for the effective treatment of postmenopausal osteoporosis.

The cellular mechanisms by which bisphosphonates inhibit osteoclast-mediated bone resorption remain to be fully elucidated. They may involve inhibition of osteoclastogenesis from immature precursor cells,⁽⁷⁻⁹⁾ alterations to the osteoclast cytoskeleton, effects on protein and membrane trafficking,^(7,10,11) and direct inhibition of resorption through induction of apoptosis in mature osteoclasts.^(9,12,13) Perhaps most importantly, at the biochemical level, it is now well established that bisphosphonates inhibit the mevalonate biosynthetic pathway in osteoclasts and macrophages.^(7,14-17) This pathway involves the synthesis of isoprenyl diphosphate intermediates, such as farnesyl diphosphate and geranylgeranyl diphosphate, which are substrates for prenyl-protein transferases catalyzing the post-translational modification of proteins. Prenylation of small GTPases with farnesyl or geranylgeranyl groups is essential for their localization to cell membranes and, in turn, for their biological function in intracellular signaling pathways.^(7,18)

The aim of this study was to investigate the magnitude and duration of the protective effect of single, increasing intravenous doses of ZOL on bone loss, bone architecture, bone formation, and bone strength in the ovariectomized (OVX) rat, an animal model of postmenopausal osteopenia.

MATERIALS AND METHODS

Skeletally-mature, 7-mo-old, virgin Wistar rats obtained from IFFA Credo (Lyon, France) were assigned to eight treatment groups (10 per group). All animals except those in the sham-operated control group were bilaterally OVX at baseline. The rats were treated with single intravenous doses of ZOL 0.8, 4, 20, 100, or 500 $\mu\text{g/kg}$, alendronate 200 $\mu\text{g/kg}$, or isotonic saline, administered into the tail vein as a slow intravenous injection over 30 s under light inhalation anesthesia with isoflurane (Forene; Abbott), 4 days before OVX. Sham-operated controls were pretreated with intravenous isotonic saline.

Animals were housed at 25°C with a 12:12-h light-dark cycle. They were fed a standard laboratory diet containing 0.8% phosphorus and 1.1% calcium (NAFAG 890). Food and water were provided ad libitum. Studies described herein were performed according to the animal permit BS575 and were approved by the Kantonales Veterinäramt (Basel, Switzerland). All animal experimentation was conducted in accordance with accepted standards of humane animal care.

Mass and density of cancellous and cortical bone in the proximal tibia metaphysis were measured in vivo at 4-wk intervals by pQCT. All pQCT measurements were carried out under inhalation anesthesia using isoflurane (Forene; Abbott) on the proximal tibia metaphysis at a distance of 4.5 mm from the proximal end of the bone on a XCT-960A tomograph (Stratec, Pforzheim, Germany).⁽¹⁹⁾ Cancellous bone architecture in the proximal tibia metaphysis was assessed in vivo at 4-wk intervals by μCT using a prototype μCT , vivaCT20 (SCANCO Medical, Bassersdorf, Switzerland).

A total of 200 slices of 26 μm thickness each were determined every time, and the resulting scan length of 5.2 mm covered the epiphysis, growth plate, and the secondary spongiosa. Blood was collected at 4-wk intervals for 32 wk from the lingual vein with a needle under light inhalation anesthesia with isoflurane to determine plasma levels of osteocalcin (rat osteocalcin IRMA-Kit; Immutopics), a serum bone marker of bone formation, and TRACP5b (Rat TRAP ELISA Kit; IDS, Frankfurt, Germany), a marker of bone resorption.

Double fluorochrome labels, alizarine complexone 20 mg/kg (Merck, Dietikon, Switzerland) and calcein 30 mg/kg (Fluka, Buchs, Switzerland), were administered subcutaneously 10 and 3 days before necropsy to evaluate bone formation dynamics in the proximal tibia metaphysis on 5- μm sections. The rats were killed at week 33 with an overdose of isoflurane, and their tibias, femora, and lumbar vertebrae were harvested for fluorochrome label-based histomorphometry and biomechanical testing. Sets of 4- μm microtome sections were cut (Microtome 2050 Supercut; Reichert-Jung, Nussloch, Germany) for evaluation of fluorochrome label-based dynamic parameters using modified QUANTIMET 600 software (Leica, Cambridge, UK). Two sections per animal were evaluated within a sampling area starting 1.0 mm from the proximal growth plate. Bone surface was measured, and mineralized surface, mineral apposition rate, and daily bone formation rate (surface referent) were calculated in accordance with the recommendations of Parfitt et al.⁽²¹⁾ Biomechanical strength was assessed by compression tests on the lumbar vertebral body and distal femoral metaphysis, a cantilever compression test on the femoral neck, and a three-point bending test on the femoral shaft. Compression testing of the femoral metaphysis has been developed and described in detail by Thomsen et al.⁽²²⁾ For the testing, the specimen was left intact (no removal of cortical bone). A 4-mm-thick section with planoparallel ends was sawn from the distal part of the femora just over the patellofemoral joint cartilage. Mechanical properties were determined in a blinded fashion using a Material Testing System (Model 5501R; Instron Corp., Canton, MA, USA).⁽²³⁾ The load-displacement curve was collected by the associated software (Merlin II; Instron Corp.). Tests were conducted using a 5-kN load cell at a constant loading rate of 6 mm/min.

Parameters (mean values $\pm$ SE) for treatment groups were compared with those of the OVX saline control group. The sham-operated control group was tested for differences from the OVX saline control group.

Statistical analyses were carried out using RS/Client 2.1.2. for Windows (Build Number 4.32; RS/1 Version 6.0.1, 1999; Domain Manufacturing Corp., Burlington, MA) or SAS software (SAS Institute, Cary, NC, USA). Data were subjected to a one-way ANOVA. Equality of variances was tested by the Levene *F*-test. Multiple-group comparisons were analyzed using the Dunnett test. All statistical tests were two-tailed, and significance was assigned at $p < 0.05$.

RESULTS

Mean body weight increased after OVX by 30.7% at 32 wk compared with only 14.7% in the sham-operated con-

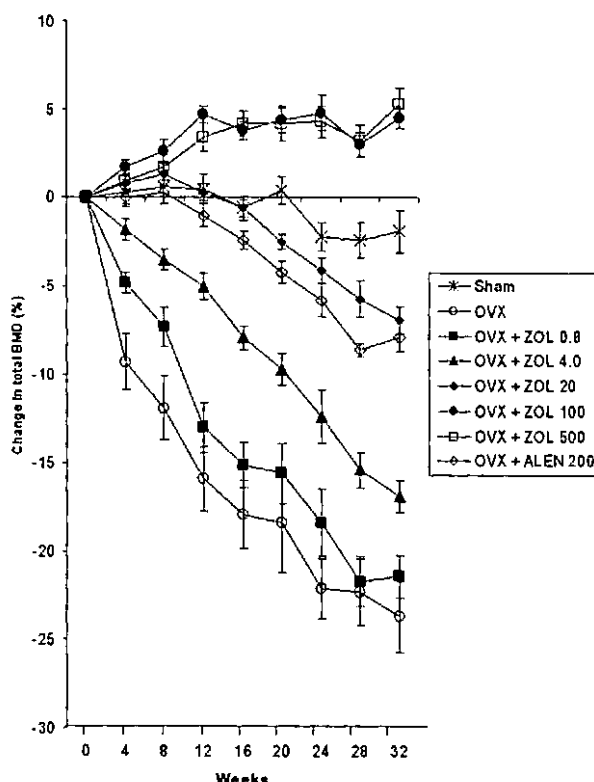


FIG. 1. Effect of zoledronic acid 0.8–500 $\mu\text{g/kg}$ and alendronate 200 $\mu\text{g/kg}$ on total BMD $\pm$ SE in OVX rats as measured by qCT in the proximal tibia metaphysis. Sham, sham operated; OVX, ovariectomized; ZOL, zoledronic acid; ALN, alendronate. Mean $\pm$ SE; $N = 10$ per group, except OVX ($N = 8$).

trols ($p < 0.01$). This gain in body weight was not significantly affected by pretreatment with ZOL or alendronate (data not shown). ZOL administered as a single intravenous dose was well tolerated.

Total, cortical, and cancellous bone mass in the proximal tibia metaphysis

Total BMD, cortical bone thickness, and cancellous BMD, as measured by pQCT, decreased rapidly after OVX (Fig. 1). This approached a plateau around 24 wk for total BMD (-22% ; $p < 0.01$ versus sham-operated controls), 20 wk for cortical bone thickness (-26% ; $p < 0.01$ versus sham-operated controls), and 28 wk for cancellous BMD (-55% ; $p < 0.01$ versus sham-operated controls).

The decline in total BMD was significantly attenuated up to 32 wk by a single pretreatment with all doses of ZOL of 4 $\mu\text{g/kg}$ and above ($p < 0.01$ versus OVX saline controls; Fig. 1). ZOL ≥ 100 $\mu\text{g/kg}$ provided complete protection against BMD loss for 32 wk, and ZOL 20 $\mu\text{g/kg}$ provided complete protection against BMD loss for 24 wk; however, significant BMD loss occurred with ZOL 20 $\mu\text{g/kg}$ at weeks 28 and 32 ($p < 0.05$ and $p < 0.01$, respectively, versus sham-operated controls). A single injection of alendronate 200 $\mu\text{g/kg}$ was of equivalent potency and duration of action to ZOL 20 $\mu\text{g/kg}$. The higher doses of ZOL had significantly higher potency versus alendronate 200 $\mu\text{g/kg}$ at week 12 onward ($p < 0.01$).

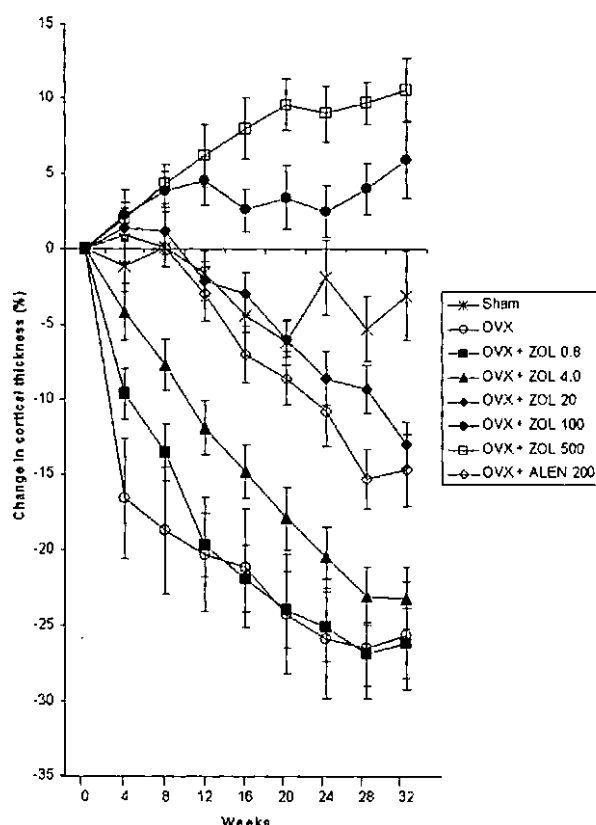


FIG. 2. Effect of zoledronic acid 0.8–500 $\mu\text{g/kg}$ and alendronate 200 $\mu\text{g/kg}$ on cortical thickness $\pm$ SE in OVX rats as measured by qCT in the proximal tibia metaphysis. Sham, sham operated; OVX, ovariectomized; ZOL, zoledronic acid; ALN, alendronate. Mean $\pm$ SE; $N = 10$ per group, except OVX ($N = 8$).

OVX-associated cortical bone thinning was significantly attenuated for 32 wk by pretreatment with all doses of ZOL ≥ 20 $\mu\text{g/kg}$ ($p < 0.01$ versus OVX saline controls; Fig. 2). ZOL ≥ 100 $\mu\text{g/kg}$ provided complete protection against cortical bone loss for 32 wk and ZOL 20 $\mu\text{g/kg}$ for 28 wk, but resulted in significant cortical bone loss at 32 wk ($p < 0.01$ versus sham-operated controls). Alendronate 200 $\mu\text{g/kg}$ was of equivalent potency and duration of action to ZOL 20 $\mu\text{g/kg}$. The higher doses of ZOL had significantly higher potency versus alendronate 200 $\mu\text{g/kg}$ at 12 wk onward ($p < 0.01$).

The OVX-associated decrease in cancellous BMD was significantly attenuated for 32 wk by pretreatment with ZOL doses ≥ 4 $\mu\text{g/kg}$ ($p < 0.01$ versus OVX saline controls; Fig. 3). Again, ZOL ≥ 100 $\mu\text{g/kg}$ provided complete protection against cancellous bone loss for 32 wk and ZOL ≥ 20 $\mu\text{g/kg}$ provided complete protection against cancellous bone loss for 28 wk; however, ZOL 20 $\mu\text{g/kg}$ resulted in significant cancellous bone loss at 32 wk ($p < 0.01$ versus sham-operated controls). Alendronate 200 $\mu\text{g/kg}$ was of equivalent potency and duration of action to ZOL 20 $\mu\text{g/kg}$. ZOL 100 and 500 $\mu\text{g/kg}$ had significantly higher potency versus alendronate 200 $\mu\text{g/kg}$ at 16 wk onward ($p < 0.01$).

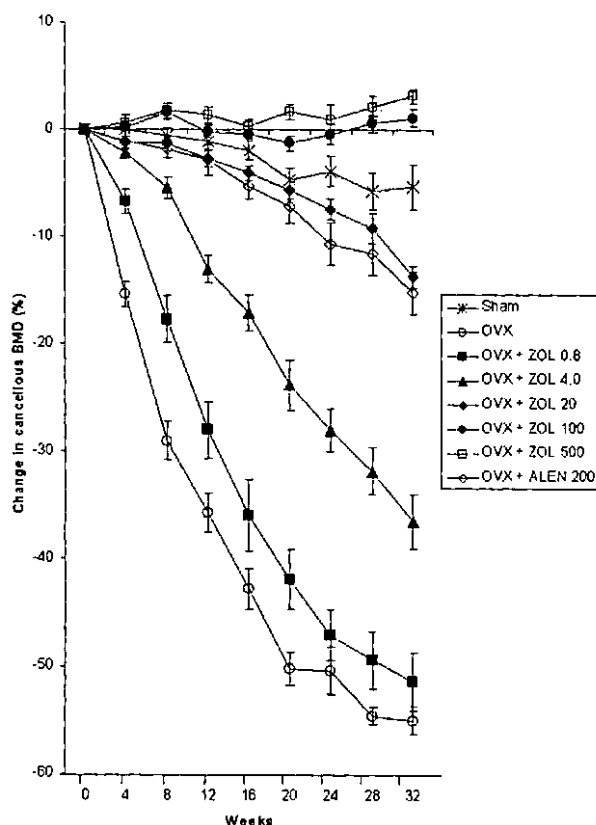


FIG. 3. Effect of zoledronic acid 0.8–500 µg/kg and alendronate 200 µg/kg on cancellous BMD ± SE in OVX rats as measured by qCT in the proximal tibia metaphysis. Sham, sham operated; OVX, ovariectomized; ZOL, zoledronic acid; ALN, alendronate. Mean ± SE; $N = 10$ per group, except OVX ($N = 8$).

Cancellous bone architecture and formation

Assessment of cancellous bone architecture by µCT showed OVX-associated decreases in trabecular bone volume, trabecular number, trabecular thickness, and connectivity density and an increase in trabecular separation. These changes were dose-dependently attenuated by ZOL 0.8–500 µg/kg at 32 wk (Table 1). The ZOL 20-µg/kg dose completely prevented these OVX-related effects, whereas ZOL 100–500 µg/kg increased trabecular bone volume, trabecular number, and connectivity density above sham-operated control levels. Alendronate 200 µg/kg was of equivalent potency and duration of action to ZOL 20 µg/kg. The higher doses of ZOL significantly increased trabecular bone volume, trabecular number, and connectivity density at 32 wk versus alendronate 200 µg/kg ($p < 0.01$), and trabecular separation was significantly lower with ZOL 500 µg/kg versus alendronate 200 µg/kg ($p < 0.05$).

Double fluorochrome label was present in bone samples from all animals at 32 wk. Assessment of fluorochrome-based dynamic cancellous bone formation parameters showed OVX did not result in significant changes in single-labeled bone surface, mineralizing surface, mineral apposition rate, or bone formation rate at 32 wk. Double-labeled surfaces increased significantly ($p < 0.05$) in OVX animals

compared with sham-operated controls. Pretreatment with ZOL 20 µg/kg had no significant effect on these bone formation parameters at 32 wk, whereas ZOL 100–500 µg/kg significantly reduced double-labeled and mineralizing bone surface, mineral apposition rate, and bone formation rate at 32 wk (Table 2). Alendronate 200 µg/kg was of equivalent potency to ZOL 100 µg/kg and suppressed all bone formation parameters, except for single-labeled bone surface, significantly more strongly than ZOL 20 µg/kg ($p < 0.01$). ZOL 500 µg/kg suppressed all bone formation parameters significantly more strongly than alendronate 200 µg/kg ($p < 0.01$).

Serum bone marker

Mean plasma osteocalcin levels rose rapidly in the first 4 wk after OVX and remained significantly elevated ($p < 0.05$ versus sham-operated controls) for the entire 32 wk. This increase in plasma osteocalcin level was significantly reduced by pretreatment with ZOL ≥ 0.8 µg/kg for 8 wk ($p < 0.01$ versus ovariectomized controls; Table 3). The effect of ZOL 20 µg/kg persisted for 24 wk, followed by a rise in osteocalcin levels, a pattern comparable to the 10-fold higher dose of ALN 200 µg/kg. At higher doses of 100 and 500 µg/kg of ZOL, osteocalcin was significantly suppressed by ~30% for the entire study duration of 32 wk.

Mean plasma TRACP5b activity rose rapidly in the first 4 wk after OVX and returned toward baseline at 24 and 32 wk (Table 4). The increase in TRACP5b was significantly reduced for 16 wk by pretreatment with ZOL ≥ 0.8 µg/kg ($p < 0.01$ versus OVX controls). The effect of ZOL 20 µg/kg was still significant at 24 wk and persisted for 32 wk at the two higher doses ($p < 0.05$ versus OVX controls), even though the levels were slowly rising toward the baseline. The two higher doses of 100 and 500 µg/kg ZOL reduced TRACP5b activity by up to 90% until week 8 after the injection, where levels started to rise slowly until the end of the study. However, at these doses, TRACP5b activity did remain significantly suppressed until week 32.

Biomechanical strength parameters

OVX significantly reduced the compression strength of the distal femoral metaphysis compared with sham-operated controls ($p < 0.05$). This was dose-dependently attenuated by ZOL 0.8–100 µg/kg (Tables 5 and 6). ZOL 100 µg/kg provided full protection of distal femoral metaphysis compression strength parameters, which was significant versus OVX controls (Table 5). ZOL 100–500 µg/kg significantly improved ultimate strength of the lumbar vertebral body in OVX animals (Table 6). Alendronate 200 µg/kg was of similar potency to ZOL 20 µg/kg in protecting against loss of compression strength of the distal femoral metaphysis and lumbar vertebral body. The higher doses of ZOL increased ultimate strength versus alendronate 200 µg/kg in both the distal femoral metaphysis and lumbar vertebral body ($p < 0.05$), and ZOL 500 µg/kg increased toughness of the distal femoral metaphysis versus alendronate 200 µg/kg ($p < 0.05$).

There were no significant changes in the strength of the femoral neck in OVX animals, either in the control or treat-

TABLE 1. EFFECT OF ZOLEDRONIC ACID 0.8–500 µg/kg AND ALENDRONATE 200 µg/kg ON TRABECULAR BONE ARCHITECTURE AS ASSESSED BY µCT OF THE PROXIMAL TIBIA METAPHYSIS AT 32 wk

	Trabecular bone volume (%)	Trabecular thickness (µm)	Trabecular number (1/mm)	Trabecular separation (µm)	Connectivity density (1/mm ³)
Sham	45.9 ± 3.1*	129 ± 5*	4.4 ± 0.13*	201 ± 10*	36.2 ± 2.1*
OVX	14.8 ± 0.8†	102 ± 5†	2.3 ± 0.08†	444 ± 17*	9.9 ± 0.9†
OVX + ZOL 0.8 µg/kg	17.8 ± 2.3†	103 ± 3†	2.5 ± 0.16†	419 ± 33*	13.2 ± 2.2†
OVX + ZOL 4 µg/kg	25.8 ± 1.2*†	106 ± 2†	3.4 ± 0.13*†	298 ± 15*	27.3 ± 2.0*
OVX + ZOL 20 µg/kg	40.1 ± 3.6*	116 ± 5†	4.7 ± 0.17*†	190 ± 12*	46.3 ± 4.0*
OVX + ZOL 100 µg/kg	56.4 ± 2.4*†	136 ± 4*	5.3 ± 0.15*†	152 ± 7*	53.0 ± 3.4*†
OVX + ZOL 500 µg/kg	58.0 ± 1.8*†	137 ± 5*	5.5 ± 0.11*†	140 ± 4*†	59.6 ± 5.2*†
OVX + ALEN 200 µg/kg	46.6 ± 4.1*	137 ± 7*	4.4 ± 0.19*	193 ± 14*	40.4 ± 2.4*

Values are mean ± SE. N = 10 per group, except OVX (N = 8).
* p < 0.01 vs. OVX.
† p < 0.01 vs. Sham.
‡ p < 0.05 vs. Sham.
Sham, sham operated; OVX, ovariectomized; ZOL, zoledronic acid; ALEN, alendronate; SEM, standard error of the mean

TABLE 2. EFFECT OF ZOLEDRONIC ACID 0.8–500 µg/kg AND ALENDRONATE 200 µg/kg ON BONE FORMATION PARAMETERS IN THE PROXIMAL TIBIA METAPHYSIS AS ASSESSED BY FLUOROCHROME LABELING WITH ALIZARINE AND/OR CALCEIN AT 32 wk

	Single-labeled bone surface (%)	Double-labeled bone surface (%)	Mineralizing surface (%)	Bone formation rate (µm/d)	Mineral apposition rate (µm/d)
Sham	53.6 ± 2.7	34.5 ± 2.6*	61.3 ± 1.4	0.373 ± 0.040	0.601 ± 0.048
OVX	49.6 ± 2.1	40.0 ± 2.0	64.8 ± 1.2	0.354 ± 0.022	0.547 ± 0.034
OVX + ZOL 0.8 µg/kg	45.9 ± 1.5	46.6 ± 1.6*†	69.5 ± 0.9*	0.394 ± 0.019	0.566 ± 0.023
OVX + ZOL 4 µg/kg	51.1 ± 0.9	40.0 ± 1.3	65.5 ± 1.3	0.356 ± 0.014	0.543 ± 0.0145
OVX + ZOL 20 µg/kg	52.6 ± 1.7	37.7 ± 1.2	64.0 ± 0.6	0.317 ± 0.016	0.494 ± 0.021‡
OVX + ZOL 100 µg/kg	45.2 ± 3.9	17.6 ± 2.6*†	40.2 ± 4.2*†	0.166 ± 0.020*†	0.403 ± 0.012*†
OVX + ZOL 500 µg/kg	31.9 ± 3.2*†	7.7 ± 1.3*†	23.7 ± 2.9*†	0.079 ± 0.012*†	0.329 ± 0.021*†
OVX + ALEN 200 µg/kg	45.1 ± 1.2‡	22.7 ± 0.9*†	45.2 ± 1.0*†	0.188 ± 0.004*†	0.416 ± 0.008†‡

Values are mean ± SE. N = 10 per group, except OVX (N = 8).
* p < 0.01 vs. OVX.
† p < 0.05 vs. OVX.
‡ p < 0.01 vs. Sham.
§ p < 0.05 vs. Sham.
Sham, sham operated; OVX, ovariectomized; ZOL, zoledronic acid; ALEN, alendronate.

ment groups (data not shown). However, OVX resulted in reduced strength parameters (including ultimate strength and elasticity) of the femoral shaft, which was dose-dependently attenuated by ZOL 0.8–200 µg/kg. ZOL ≥20 µg/kg and alendronate significantly improved the strength, stiffness, and elasticity of the femoral shaft versus that of sham-operated controls (Table 7). Full protection of parameters was seen with ZOL 100–500 µg/kg and alendronate 200 µg/kg.

DISCUSSION

The results presented here show that the duration of the protective effect of a single intravenous dose of ZOL 0.8–500 µg/kg on loss of cortical and cancellous bone mass and disruption of cancellous bone architecture in OVX rats is dose dependent, with ZOL 100–500 µg/kg providing protection for 32 wk. Furthermore, the magnitude of the protective effect of ZOL on bone mass is also dose related, with ZOL 20 µg/kg offering full bone protection, whereas only partial protection was seen at lower doses. Compared with alendronate, currently the most widely prescribed bi-

sphosphonate therapy, ZOL shows 10-fold higher potency in preventing OVX-associated bone loss, with higher doses of ZOL (100–500 µg/kg) showing significantly higher potency and duration of action than alendronate 200 µg/kg, without any indication of deterioration of mechanical parameters.

Although there are few other reports of preclinical studies on the sustained inhibition of bone resorption after a single administration of bisphosphonate, similar long-term effects have been observed in patients with Paget's disease and osteoporosis treated with 2- to 5-day intravenous loading doses of alendronate and neridronate.^(24–26) Therefore, prolonged efficacy is exhibited by several of the nitrogen-containing bisphosphonates but, because of its high therapeutic ratio, ZOL has the unique advantage that an effective dose can be safely administered as a convenient single 15-min infusion.

After 32 wk of treatment with ZOL 20 µg/kg, dynamic bone formation was similar to that of sham-operated controls, indicating normal bone turnover was restored at this time point. Plasma osteocalcin levels did not suggest that bone formation was markedly suppressed at any time at

TABLE 3. EFFECT OF ZOLEDRONIC ACID 0.8–500 µg/kg AND ALENDRONATE 200 µg/kg ON PLASMA OSTEOCALCIN IN OVX RATS

	Plasma osteocalcin (ng/ml)								
	Week 0	Week 4	Week 8	Week 12	Week 16	Week 20	Week 24	Week 28	Week 32
Sham	14.5 ± 0.9	12.0 ± 0.7*	11.2 ± 0.5*	12.7 ± 1.1*	11.5 ± 1.1*	13.7 ± 1.0†	11.3 ± 1.4†	9.1 ± 1.2‡	9.7 ± 1.4*
OVX	13.1 ± 0.4	24.1 ± 1.4‡	20.8 ± 1.0‡	17.9 ± 1.1‡	20.6 ± 1.3‡	18.5 ± 1.3‡	16.0 ± 1.0‡	14.0 ± 1.0‡	14.8 ± 1.8‡
OVX + ZOL 0.8 µg/kg	13.4 ± 0.8	17.4 ± 1.1*‡	15.7 ± 1.1*‡	16.2 ± 0.7‡	18.5 ± 0.8‡	17.5 ± 0.7‡	15.1 ± 0.8‡	14.6 ± 0.6‡	14.3 ± 0.7‡
OVX + ZOL 4 µg/kg	13.3 ± 0.6	15.7 ± 1.2*‡	14.6 ± 0.8*‡	13.9 ± 0.4†	17.1 ± 1.1*‡	13.6 ± 0.8*	14.5 ± 1.2	14.8 ± 0.9‡	13.6 ± 1.0‡
OVX + ZOL 20 µg/kg	12.4 ± 0.4	14.9 ± 1.0*	12.6 ± 0.6*	11.6 ± 0.4*	13.1 ± 1.0*	14.7 ± 1.8†	11.4 ± 1.4*	12.4 ± 2.4	16.5 ± 1.5†
OVX + ZOL 100 µg/kg	14.1 ± 0.8	12.0 ± 0.4*	10.2 ± 0.5*	9.0 ± 0.4*‡	9.5 ± 0.4*	9.8 ± 0.6*‡	6.7 ± 0.7*‡	6.7 ± 0.6‡	7.5 ± 0.5*
OVX + ZOL 500 µg/kg	12.5 ± 0.6	12.1 ± 0.6*	9.5 ± 0.5*	8.5 ± 0.4*‡	9.2 ± 0.9*	9.5 ± 0.7*‡	9.6 ± 0.7*	9.1 ± 0.5‡	8.0 ± 0.4*
OVX + ALEN 200 µg/kg	13.3 ± 0.4	14.8 ± 0.7*	12.1 ± 0.5*	12.4 ± 0.7*	13.7 ± 0.6*	13.9 ± 0.8*	14.2 ± 0.7	12.5 ± 0.6	13.2 ± 0.7‡

Values are mean ± SE. N = 10 per group, except OVX (N = 8).
* p < 0.01 vs. OVX.
† p < 0.05 vs. OVX.
‡ p < 0.01 vs. Sham.
§ p < 0.05 vs. Sham.
Sham, sham operated; OVX, ovariectomized; ZOL, zoledronic acid; ALEN, alendronate.

TABLE 4. EFFECT OF ZOLEDRONIC ACID 0.8–500 µg/kg AND ALENDRONATE 200 µg/kg ON PLASMA TRACP5b IN OVX RATS

	Plasma TRACP5b (U/liter)						
	Week 0	Week 4	Week 8	Week 12	Week 16	Week 24	Week 32
Sham	5.62 ± 0.57	5.83 ± 0.60*	4.89 ± 0.28*	5.09 ± 0.31*	5.78 ± 0.34*	6.05 ± 0.34	5.92 ± 0.68
OVX	5.17 ± 0.34	9.17 ± 0.57	12.41 ± 1.02	11.32 ± 1.17	10.24 ± 0.88	7.25 ± 0.58	6.08 ± 0.56
OVX + ZOL 0.8 µg/kg	5.16 ± 0.62	2.43 ± 0.37*†	3.03 ± 0.35*†	3.75 ± 0.22*†	5.38 ± 0.69*	6.24 ± 0.50	6.70 ± 0.38
OVX + ZOL 4 µg/kg	5.64 ± 0.35	1.40 ± 0.26*†	2.55 ± 0.29*†	3.40 ± 0.25*†	4.02 ± 0.41*†	5.09 ± 0.69†	5.58 ± 0.38
OVX + ZOL 20 µg/kg	5.58 ± 0.47	0.79 ± 0.17*†	0.99 ± 0.22*†	2.28 ± 0.24*†	3.55 ± 0.33*†	5.26 ± 0.33*	5.46 ± 0.40
OVX + ZOL 100 µg/kg	5.64 ± 0.68	0.79 ± 0.12*†	1.13 ± 0.25*†	1.59 ± 0.30*†	3.38 ± 0.41*†	3.93 ± 0.48*†	4.61 ± 0.38‡
OVX + ZOL 500 µg/kg	5.65 ± 0.42	0.43 ± 0.05*†	0.57 ± 0.20*†	1.56 ± 0.35*†	2.42 ± 0.20*†	2.60 ± 0.26*†	3.10 ± 0.27*†
OVX + ALEN 200 µg/kg	5.97 ± 0.35	1.05 ± 0.09*†	1.27 ± 0.12*†	1.46 ± 0.17*†	3.75 ± 0.26*†	5.10 ± 0.64‡	4.72 ± 0.49

Values are mean ± SE. N = 10 per group, except OVX (N = 8).
* p < 0.01 vs. OVX.
† p < 0.01 vs. Sham.
‡ p < 0.05 vs. OVX.
Sham, sham operated; OVX, ovariectomized; ZOL, zoledronic acid; ALEN, alendronate.

doses of up to 20 µg/kg. Alendronate showed relatively potent suppression of dynamic bone formation, with 200 µg/kg equivalent to ZOL 100 µg/kg and significantly greater suppression than ZOL 20 µg/kg, which was equivalent to alendronate 200 µg/kg at attenuating BMD loss. High-dose ZOL (100–500 µg/kg) reduced bone formation below the level of sham-operated controls; however, the degree of inhibition of plasma osteocalcin did not exceed 50%, indicating bone formation continued, even at doses 5-fold higher than the equivalent human dose (500 µg/kg). The bone resorption marker TRACP5b indicated transient suppression of elevated osteoclast activity by ZOL relative to OVX rats even at the lowest dose of 0.8 µg/kg, whereas at 100–500 µg/kg, the effect was significant relative to the OVX control for the entire duration of the study of 32 wk. Intravenous injection of ZOL produced significant dose-related improvements in mechanical properties of the

femoral shaft (20–500 µg/kg), the metaphysis, and lumbar vertebral body (100 and 500 µg/kg) in OVX rats. ZOL can fully prevent loss of bone strength after OVX, and at doses of 100 or 500 µg/kg, equivalent to 5- or 25-mg doses, respectively, in humans, strength parameters were at least 10% higher than those in sham-operated controls. This increase in bone strength could be at least in part caused by a shift in the mineral density profile resulting in increased secondary mineralization as a consequence of lower bone turnover.^(27–30) More recently, Durchschlag et al.⁽³¹⁾ also suggested that long-term treatment of osteoporotic women with risedronate affects the bone mineral maturity/crystallinity and collagen cross-link ratio with positive consequences on tissue aging. In our study, structural analysis of cancellous bone by µCT indicated that the higher doses of 100 and 500 µg/kg ZOL may have protected against the age-related decrease in trabecular number while having

TABLE 5. EFFECT OF ZOLEDRONIC ACID 0.8–500 µg/kg AND ALENDRONATE 200 µg/kg ON COMPRESSION STRENGTH PARAMETERS OF THE DISTAL FEMORAL METAPHYSIS

	Ultimate strength (N/mm ²)	Elastic modulus (MPa)	Toughness (MJ/m ²)
Sham	31.60 ± 2.58*	872.36 ± 36.30*	1.09 ± 0.16*
OVX	20.98 ± 0.90†	680.35 ± 33.85‡	0.69 ± 0.06‡
OVX + ZOL 0.8 µg/kg	16.68 ± 2.04†	495.45 ± 67.26*	0.54 ± 0.08†
OVX + ZOL 4 µg/kg	21.63 ± 1.35‡	653.11 ± 55.17‡	0.81 ± 0.08
OVX + ZOL 20 µg/kg	23.24 ± 1.73‡	715.49 ± 31.06	0.81 ± 0.10
OVX + ZOL 100 µg/kg	36.37 ± 2.82*	984.67 ± 63.10*	1.29 ± 0.11*
OVX + ZOL 500 µg/kg	35.50 ± 2.22*	885.70 ± 67.95	1.50 ± 0.13
OVX + ALEN 200 µg/kg	27.44 ± 1.54	812.30 ± 64.95	0.96 ± 0.08

Values are mean ± SE. *N* = 10 per group, except OVX (*N* = 8).
* *p* < 0.05 vs. OVX.
† *p* < 0.01 vs. Sham.
‡ *p* < 0.05 vs. Sham.
Sham, sham operated; OVX, ovariectomized; ZOL, zoledronic acid; ALEN, alendronate.

TABLE 6. EFFECT OF ZOLEDRONIC ACID 0.8–500 µg/kg AND ALENDRONATE 200 µg/kg ON COMPRESSION STRENGTH PARAMETERS OF THE LUMBAR VERTEBRAL BODY

	Ultimate strength (N/mm ²)	Elastic modulus (MPa)	Toughness (MJ/m ²)
Sham	28.53 ± 2.10	667.85 ± 56.10	1.06 ± 0.13
OVX	25.59 ± 0.71	623.30 ± 36.33	0.94 ± 0.08
OVX + ZOL 0.8 µg/kg	20.03 ± 1.14*	423.19 ± 36.11	0.71 ± 0.09
OVX + ZOL 4 µg/kg	26.07 ± 2.50	599.64 ± 66.82	0.88 ± 0.09
OVX + ZOL 20 µg/kg	28.41 ± 2.29	851.49 ± 129.20	0.87 ± 0.09
OVX + ZOL 100 µg/kg	37.16 ± 2.95**	820.05 ± 120.94	1.50 ± 0.14**
OVX + ZOL 500 µg/kg	34.28 ± 1.96*	767.59 ± 74.34	1.25 ± 0.12
OVX + ALEN 200 µg/kg	28.13 ± 1.40	742.11 ± 74.38	0.99 ± 0.072

Values are mean ± SE. *N* = 10 per group, except OVX (*N* = 8).
* *p* < 0.05 vs. Sham.
† *p* < 0.05 vs. OVX.
Sham, sham operated; OVX, ovariectomized; ZOL, zoledronic acid; ALEN, alendronate.

TABLE 7. EFFECT OF ZOLEDRONIC ACID 0.8–500 µg/kg AND ALENDRONATE 200 µg/kg ON BENDING STRENGTH PARAMETERS OF THE FEMORAL SHAFT

	Ultimate strength (N/mm ²)	Elastic modulus (MPa)	Toughness (MJ/m ²)
Sham	189.59 ± 5.35*	6793.99 ± 317.99*	6.55 ± 0.44
OVX	170.52 ± 4.61†	5359.84 ± 263.77‡	5.90 ± 0.36
OVX + ZOL 0.8 µg/kg	150.23 ± 6.02‡	5489.08 ± 260.56†	5.08 ± 0.26
OVX + ZOL 4 µg/kg	186.84 ± 5.69	6395.25 ± 345.45*	6.62 ± 0.39
OVX + ZOL 20 µg/kg	191.21 ± 7.17*	6586.81 ± 301.29*	6.49 ± 0.35
OVX + ZOL 100 µg/kg	205.97 ± 5.19*	6398.00 ± 263.61*	7.40 ± 0.46*
OVX + ZOL 500 µg/kg	217.05 ± 4.35**	7762.48 ± 232.24*	7.56 ± 0.47*
OVX + ALEN 200 µg/kg	201.57 ± 4.11*	6437.88 ± 324.97*	8.06 ± 0.57**

Values are mean ± SE. *N* = 10 per group, except OVX (*N* = 8).
* *p* < 0.05 vs. OVX.
† *p* < 0.05 vs. Sham.
‡ *p* < 0.01 vs. Sham.
Sham, sham operated; OVX, ovariectomized; ZOL, zoledronic acid; ALEN, alendronate.

only a minor effect on trabecular thickness. Therefore, preservation of connectivity may also have contributed to the improvement in mechanical parameters observed in our study.

In conclusion, in this animal model of postmenopausal osteopenia, a single dose of 100 µg/kg ZOL equivalent to the human 5-mg dose suppresses bone resorption, protects against cancellous and cortical bone loss, and prevents the deterioration of mechanical parameters for up to 32 wk. These data support the use of an annual intravenous ZOL dosing regimen for the treatment of osteoporosis.

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