

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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Señores

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

DIVISIÓN DE NUEVAS CREACIONES

E. S. D.

Re: Expediente N°. 11-030.452

Solicitud de Patente de Invención titulada: "PROCESOS PARA LA PREPARACIÓN DEL METIL-ÉSTER DEL ÁCIDO 4-OXO-OCTAHIDRO-INDOL-1-CARBOCÍCLICO Y DERIVADOS DEL MISMO"

Solicitante: NOVARTIS AG

Nuestra Ref: P2011/000026

RESPUESTA AL OFICIO No. 15990, NOTIFICADO POR FIJACIÓN EN LISTA No. 034
DE FECHA 27 DE AGOSTO DE 2013
(PRIMER EXAMEN DE FONDO)

CARLOS R. OLARTE, mayor de edad y vecino de Bogotá, identificado tal como aparece al pie de mi firma, en mi carácter de apoderado de la sociedad solicitante, me permito manifestar lo siguiente ante el auto emanado por ese Despacho, dentro del plazo legal de prórroga previsto en el artículo 45 de la Decisión 486, en los siguientes términos:

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 voluntariamente el 28 de julio de 2011.

Así entonces, el Capítulo Reivindicatorio Modificado contiene los siguientes cambios:

- se eliminan las antiguas Reivindicaciones 4, 5 y 9;
- en la Reivindicación 2 se limita R¹ a cloro, bromo o yodo, metasulfonato o fluorosulfonato;

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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 voluntariamente el 28 de julio de 2011.

Así entonces, el Capítulo Reivindicatorio Modificado contiene los siguientes cambios:

- se eliminan las antiguas Reivindicaciones 4, 5 y 9;
- en la Reivindicación 2 se limita R¹ a cloro, bromo o yodo, metasulfonato o fluorosulfonato;

- las nuevas Reivindicaciones 4 y 8 corresponden a las antiguas Reivindicaciones 6 y 11, respectivamente, en donde se limita R¹ a cloro, bromo o yodo, metasulfonato o fluorosulfonato;
- las nuevas Reivindicaciones 5 y 6 corresponden a las antiguas Reivindicaciones 7 y 8, respectivamente.

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.

2. a- El Examinador señala que la Reivindicación 5 no es clara y no se encuentra sustentada en el Capítulo Descriptivo, ya que reclama una composición en términos del proceso. Adicionalmente el Examinador señala que las composiciones se deben definir indicando los ingredientes, activos y los excipientes farmacéuticamente aceptables junto con las proporciones de cada uno, adicionalmente dichas composiciones no se encuentran soportadas con ejemplos concretos dentro del Capítulo Descriptivo. Finalmente el Examinador sugiere eliminar dicha reivindicación.

b- En respuesta a lo anterior, la antigua Reivindicación 5 fue eliminada del Capítulo Reivindicatorio Modificado adjunto, por lo que considero superada la objeción mencionada anteriormente.

3. a- El Examinador señala que las Reivindicaciones 2, 6, 8 y 11 no son concisas porque la definición de R¹ es general, por lo tanto, el Examinador sugiere restringir la solicitud a una generalización razonable del tipo de sustituyentes que han sido probados, es decir, que se encuentran sustentados de acuerdo con la siguiente tabla:

RADICAL	SOPORTE DE EJEMPLOS PRESENTADOS
R1	Cloro

b- En primer lugar, me permito llamar respetuosamente la atención del Examinador sobre el Capítulo Reivindicatorio Modificado adjunto, en especial, sobre la drástica limitación de R¹, en la cual se limitó dicho radical exclusivamente a cloro, bromo o yodo, metanosulfonato o fluorosulfonato, lo cual encuentra soporte en la página 3, líneas 12-16 del Capítulo Descriptivo.

En adición a lo anterior, me permito llamar igualmente la atención sobre las disposiciones de la Guía para el Examen de Solicitudes de Patentes de Invención y Modelos de Utilidad, en donde se establece que si una reivindicación comprende un número muy grande de alternativas, se presentará un requerimiento al solicitante que sugiera restringir la solicitud a una razonable generalización de la materia que ha sido desarrollada o probada, **pero no se trata de requerir para que se restrinja sólo a los ejemplos**.¹ En el presente caso, el Examinador argumenta que el alcance de R¹ no se encuentra debidamente reflejado en los ejemplos de la presente invención, sugiriendo al solicitante a limitar **únicamente** a la materia ejemplificada, lo cual va en contravía de las disposiciones de la guía anteriormente citada.

Asimismo, no puede olvidarse que un inventor está en su derecho de reclamar protección para la **totalidad de un concepto inventivo desarrollado, sin importar la amplitud de éste**, siempre y cuando se conserve el principio de unidad de invención y no haya arte previo que afecte su patentabilidad. Es decir, una reivindicación puede ser tan amplia como se requiera para así poder enmarcar la totalidad del concepto inventivo desarrollado y no solamente las modalidades preferidas o específicamente ejemplificadas en la solicitud. Asimismo, la posibilidad de obtener la invención en diversas modalidades es una práctica aceptada a nivel mundial en el ámbito de las patentes, ya que como el Examinador debe saberlo, una patente de invención **no se limita a una única modalidad**, si no que cubre un concepto inventivo global que abarca diferentes modalidades (diversas materializaciones de la invención) ligadas por un efecto técnico común que finalmente delimita el alcance de la invención.

En atención a lo anterior, y como ya lo mencioné, no existe requerimiento legal alguno que determine que los Ejemplos deban ilustrar todas y cada una de las variaciones de los diferentes sustituyentes del compuesto central de la presente invención que se encuentran dentro del alcance de la invención, ya que lo fundamental es que el Capítulo Descriptivo proporcione la información necesaria para que la persona medianamente versada en la materia pueda reproducir la invención sin tener que acudir a experimentación adicional excesiva. Vale la pena recordarle al Examinador que la función de los ejemplos en el Capítulo Descriptivo es **estrictamente ilustrativa** y de ninguna manera puede considerarse como el único alcance reivindicable posible.

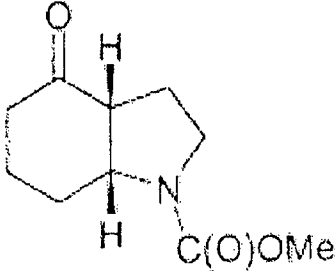
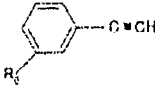
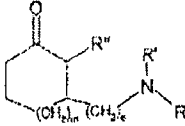
Así, considero respetuosamente que el Capítulo Reivindicatorio Modificado adjunto cumple con los requisitos de claridad y concisión establecidos en la Decisión 486, y a la luz del MAP y la Guía de la SIC, permitiendo a la persona medianamente versada en la materia comprender

¹ Guía para Examen de Solicitudes de Patentes de Invención y Modelo de utilidad. Superintendencia de Industria y Comercio. 2012. Sección 3.3.4.1 (disponible en http://api.sic.gov.co/Documentos/Guia_Examen_Patentes.pdf)

inequívocamente el alcance de la materia reclamada y el cual corresponde a una generalización razonable de la materia ilustrada en el Capítulo Descriptivo.

4. a- El Examinador señala que la patente WO03/047581 (D1) afecta la novedad de las Reivindicaciones 1 a 11 de la presente invención.

El Examinador compara las características esenciales de la Reivindicación 1 de la siguiente manera:

Características esenciales	D1
Proceso para la producción de un compuesto de fórmula (I), el cual comprende hacer reaccionar: 3-etinil-tolueno Con un compuesto de fórmula (II): 	Proceso para la producción de un compuesto de fórmula (I), el cual comprende hacer reaccionar, un compuesto de fórmula (III)  Donde Ro= alquilo C1-4 (Pág. 1) y (Pág. 2, compuesto de fórmula (III)) Con un compuesto de fórmula (II):  (Pág. 2) Donde: m= 1 (Pág. 1) n= 1 (Pág. 1) R= C(O)OR3 (Pág. 1) R3= alquilo C1-4 (Pág. 1) R' y R''= en conjunto forman un grupo -CH2-(CH2)p (Pág. 1) p= 1 (Pág. 1)

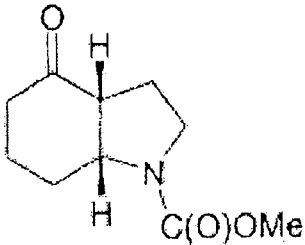
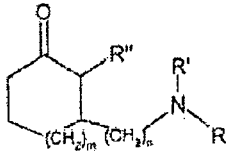
A partir de lo anterior, el Examinador afirma que las características esenciales del proceso para la producción de un compuesto de Fórmula I de la Reivindicación 1 han sido previamente divulgadas por D1, el cual enseña un proceso para producir un compuesto de Fórmula (I), el cual comprende hacer reaccionar un compuesto de fórmula (III) con un compuesto de fórmula (II). De acuerdo con lo anterior, el Examinador concluye que la Reivindicación 1 no es novedosa.

En cuanto a las Reivindicaciones 2-9 el Examinador señala que no mencionan ninguna característica que haga nueva la Reivindicación 1, por lo que tampoco se consideran nuevas.

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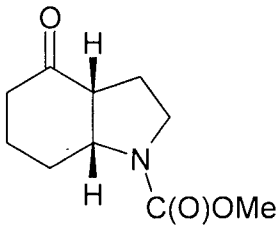
OLARTE MOURE & ASOCIADOS LTDA.

Por otro lado, respecto a la Reivindicación 10, el Examinador compara las características esenciales de dicha reivindicación con D1 de la siguiente forma:

Características esenciales	D1
Compuesto de fórmula (II): 	Con un compuesto de fórmula (II):  (Pág. 2) Donde: m= 1 (Pág. 1) n= 1 (Pág. 1) R= C(O)OR3 (Pág. 1) R3= alquilo C1-4 (Pág. 1) R' y R''= en conjunto forman un grupo -CH2-(CH2)p (Pág. 1) p= 1 (Pág. 1)

El Examinador señala que las características esenciales del compuesto de la Reivindicación 10 han sido previamente divulgadas por D1, el cual enseña un compuesto de fórmula (II) que contiene m= 1, n=1, R= C(O)OR₃, R₃= alquilo C₁₋₄, R' y R''=en conjunto forman un grupo -CH₂-(CH₂)p y p=1. De acuerdo con lo anterior, la materia reivindicada no es nueva.

b- Con respecto de la novedad, en primer lugar me permito señalar que la presente invención reclama un procedimiento para la producción del éster metílico del ácido (3aR, 4S, 7aR)-4-hidroxi-4-m-toliletinil-octahidro-indol-1-carboxílico (I), (2-cloro-etil)-(3-oxo-ciclohexil)-alquil-éster del ácido carbámico (II), y de enantiómeros de 1-carbalcoxi-4-ceto-perhidro-indol (III), en donde dicho procedimiento comprende hacer reaccionar 3-etinil-tolueno con un compuesto de la Fórmula (II), o una sal del mismo:



(II)

De acuerdo con lo anterior el reactivo de partida del procedimiento corresponde al éster metílico del ácido 4-oxo-octahidro-indol-1-carboxílico.

Ahora bien, con respecto al arte previo citado, me permito llamar la atención del Examinador sobre el ejemplo 1 de D1, tal y como se describe en las etapas e) a g) de dicho ejemplo, las cuales muestran el uso específicamente de 1-etinil-3-metil-benceno como el compuesto III, el cual se

hace reaccionar necesariamente con el éster terc-butílico de ácido 4-oxo-octahidro-indol-1-carboxílico. En particular, D1 lo señala de la siguiente manera:

(e) *Una solución de 1-etinil-3-metil-benceno (3.248g; 28 mmol) en THF (168 ml) se enfría a -20°C, una solución de butillitio (17.5ml; 28 mmol; 1.6M en hexano) es adicionado. La mezcla es agitada a -20°C durante 2 horas posteriormente una solución de **éster terc-butílico de ácido 4-oxo-octahidro-indol-1-carboxílico** (3.346g; 14 mol) en THF (70ml) es adicionado y la mezcla de reacción es además agitada entre 0-5°C (...)* (mi énfasis)

Teniendo en cuenta lo anterior, D1 se diferencia de la presente invención debido al uso de un éster terc-butílico como reactivo de partida, en lugar del éster metílico correspondiente, tal y como en la presente invención.

Así las cosas, D1 no divulga un procedimiento para la obtención del éster metílico del ácido (3aR, 4S, 7aR)-4-hidroxi-4-m-toliletinil-octahidro-indol-1-carboxílico (I), (2-cloro-etil)-(3-oxo-ciclohexil)-alquil-éster del ácido carbámico (II), y de enantiómeros de 1-carbalcoxi-4-cetoperhidro-indol (III), con las características estructurales específicamente aquí reclamadas, tal como se mostró anteriormente. De hecho, D1 divulga reactivos de reacción competamente diferentes. Por tal razón, el procedimiento de la Reivindicación 1 no se encuentra anticipado por D1.

Respecto a la Reivindicación 10, como mencioné anteriormente, el reactivo de la presente invención corresponde al éster metílico del ácido 4-oxo-octahidro-indol-1-carboxílico, mientras que D1 usa como reactivo el éster terc-butílico de ácido 4-oxo-octahidro-indol-1-carboxílico. En este sentido, la Reivindicación 10 de la presente invención resulta novedosa frente al arte previo citado por el Examinador.

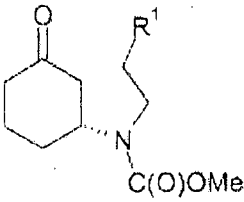
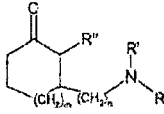
Así las cosas, vale la pena recordar que para fundamentar una afectación válida contra la novedad de una invención, el arte previo debe divulgar explícitamente todas y cada una de las características de la invención.² Es más, el Tribunal Andino de Justicia (TAJ) ha determinado que para establecer una objeción válida contra la novedad, la divulgación del arte previo debe ser lo suficientemente detallada para que una persona versada en la materia pueda usar dicha

² Manual Andino de Patentes. (2004). Sección 9.4. (disponible en <http://www.comunidadandina.org/public/patentes.pdf>).

información para reproducir y explotar la invención.³ En el presente caso, D1 no divulga explícitamente las características del procedimiento de la presente invención, por tal razón la materia aquí reclamada es novedosa frente a D1.

Con respecto a las Reivindicaciones 2 a 9, dichas reivindicaciones al ser dependientes de la Reivindicación 1, la cual es novedosa, también son novedosas. En este punto me permito citar la Guía para el Examen de patentes, la cual señala que “una reivindicación dependiente es patentable, si la reivindicación independiente de la cual se deriva es patentable, por lo cual, en este caso, no hay que hacer búsqueda para la reivindicación dependiente.”⁴

5. a- El Examinador señala que D1 afecta el nivel inventivo de la Reivindicación 11. El Examinador realiza la siguiente comparación:

Características esenciales	D1
Compuesto de fórmula (III):  Dónde: R1= halógeno	Con un compuesto de fórmula (II):  (Pág. 2) Donde: m= 1 (Pág. 1) n= 1 (Pág. 1) R= C(O)OR3 (Pág. 1) R3= alquilo C1-4 (Pág. 1) R'= hidrógeno (Pág. 1) R''= hidrógeno (Pág. 1) p= 1 (Pág. 1)

El Examinador argumenta que la diferencia entre la Reivindicación 11 y D1 consiste en que R¹ en el compuesto reclamado corresponde a un halógeno, mientras que el compuesto divulgado en D1 corresponde a un hidrógeno. El efecto que logra incluir el radical R¹ como un halógeno dentro de la estructura del compuesto reclamado consiste en mejorar el proceso de producción del compuesto de Fórmula (I).

De esta forma, el Examinador señala que el problema técnico a resolver es cómo modificar el proceso de producción del compuesto de fórmula (I) para aumentar su rendimiento. Sin embargo, los rendimientos de D1 se encuentran por encima del 90%, y la presente invención no muestra rendimientos para sus procesos, por lo que no se observa un efecto sorprendente en la invención, por lo tanto la persona medianamente versada en la materia incluiría el flúor dentro de los compuestos reclamados en D1 y así llegaría al objeto de la presente invención, por lo que el Examinador la considera obvia.

³ Proceso 06-IP-89 (<http://intranet.comunidadandina.org/Documentos/Procesos/209-IP-2005.doc>); y Proceso 36-IP-2004 (<http://www.notinet.com.co/serverfiles/servicios/archivos/30jul04/ca36-04.htm>)

⁴ Guía para examen de solicitud de patentes de invención y modelos de utilidad (2012). Sección 2.6.4.2 (disponible en http://api.sic.gov.co/Documentos/Guia_Examen_Patentes.pdf)

b- Con relación al nivel inventivo de la presente invención, me permito señalar respecto del arte previo citado, que D1 menciona una serie de derivados de acetileno que tienen actividad hacia los receptores de glutamato metabotrópicos humanos (mGluRs) y un procedimiento para su síntesis que implica la hidrogenación de un 1,5,6,7-tetrahidroindol-4-ona. Es decir, el procedimiento descrito en D1 comienza con la protección del nitrógeno del indol con el grupo carbamato del t-butilo, previo a una etapa de hidrogenación para formar el hidroindol. Posteriormente, reacciona con 1-etinil-3-metil-benceno, antes de que el grupo protector carbamato del t-butilo se elimine y se sustituya con el grupo carbamato del metilo.

En consecuencia, D1 necesariamente incluye un carbamato de t-butilo en su proceso, lo cual requiere una **etapa de hidrogenación**. Dicha etapa de hidrogenación es engorrosa, adicionalmente proporciona bajo rendimiento, tiene poca selectividad para el estereoisómero (3aR, 4S, 7aR) y se forman subproductos no deseados. De hecho, D1 señala explícitamente en su Ejemplo 1, etapa (b) lo siguiente:

b) *tert-butil ester del 1,5,6,7-tetrahidro-indol-4-ona-1-ácido carboxílico (60g; 255mmol) y 15g de Pt en carbono al 5% (adicionado en tres porciones de 5g cada 24h, 48, 72h) en 1 l de metanol es **hidrogenado** (1 bar) a temperatura ambiente bajo agitación por 92 h. (mi énfasis)*

Por el contrario, la presente invención **no implica una etapa de hidrogenación**. En lugar de ello, el compuesto de fórmula (II) se forma por el cierre del anillo estereoespecífico, **sin pasar por la etapa de hidrogenación**. Incluso en el Capítulo Descriptivo de la presente invención (página 1, líneas 9 a 25 y página 2, líneas 1 a 4), hace referencia a D1 y señala de forma clara los problemas que conlleva el procedimiento de reacción allí descrito. De esta forma, no resulta obvio para una persona medianamente versada en la materia eliminar la etapa de hidrogenación a partir de las enseñanzas de D1, por el contrario, dicha persona estaría motivada necesariamente a utilizar dicha etapa en el procedimiento. En este sentido, el Examinador deja de lado una de las características esenciales del procedimiento de la presente invención, el cual **NO** incluye una etapa de hidrogenación, y solo centra su análisis en uno de los intermediarios de reacción, analizando de forma sesgada y limitada la presente invención.

Ahora bien, la persona medianamente versada en la materia siguiendo las enseñanzas de D1 no solo no habría podido derivar de manera obvia la presente invención sino que también habría tenido que encontrar las condiciones de reacción adecuadas para formar el compuesto de fórmula

(II), principalmente en su conformación cis, es decir, (S, S) o (R, R), las cuales de ninguna manera se encuentran sugeridas en D1. De hecho, las condiciones de reacción de la presente invención son tales que la conformación trans es menos de 1.0%.

Por tal razón, a continuación se muestran los datos analíticos para el compuesto AFQ056-D6 medidos por HPLC-UV. En particular, los datos adjuntos representan el isómero cis de un compuesto de fórmula (II) y NP son los subproductos, así:

Lote	Pureza AFQ056-D6 [%A]	NP en RRT 1.1 [%A]
1	97.9	1.3
2	97.9	1.4
3	98	1.2
4	97.6	0.83
5	97.9	0.77
6	97.8	0.76
7	98.2	0.72
8	98.3	0.70
9	97.9	0.76
10	97.9	0.77
11	98.5	0.74

Así las cosas, ninguno de los procedimientos descritos en el arte previo citado por el Examinador dan como resultado un grado de pureza del producto obtenido tal y como se obtiene con el procedimiento de la presente invención.

De esta forma, no hay enseñanzas en el arte previo citado, que hubiera llevado a la persona medianamente versada en la materia, que se enfrentan con el problema técnico objetivo de sintetizar un compuesto enantioméricamente puro de fórmula (I), a omitir la etapa de hidrogenación de D1 con la expectativa de éxito de llegar a un proceso que tiene como producto final un compuesto enantioméricamente puro de fórmula (I). De acuerdo con lo anterior, la materia objeto reivindicada en la presente invención resulta inventiva sobre el arte previo citado por el Examinador.

Adicionalmente, los ejemplos en el Capítulo Descriptivo de la presente solicitud describen cómo se han obtenido los compuestos reivindicados. En particular, un compuesto de fórmula (IV) se obtuvo con el Ejemplo 1; un compuesto de fórmula (II) se obtuvo con los Ejemplos 6 y 7; un

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compuesto de fórmula (III) se obtuvo con el Ejemplo 16 (anexo al presente memorial se adjunta la reacción del nuevo compuesto de fórmula (II) con 3 etinil-tolueno, tal y como se reclama en la Reivindicación 1 (ver Anexo 1)).

En este punto vale la pena señalar que el MAP establece que para que pueda negarse la existencia de nivel inventivo, es necesario no solamente que la combinación de las enseñanzas pueda hacerse, sino también que exista una sugerencia o razón tal, que lleve al técnico con conocimientos medios a combinar las enseñanzas de los documentos⁵. Además, el Manual Andino de Patentes también establece que las invenciones en el campo de la química tienen nivel inventivo cuando los compuestos tienen una **estructura inesperada** (no derivable por la persona medianamente versada en la materia) o cuando presentan un **efecto técnico inesperado o mejorado impredecible del arte previo**⁶. Por tal razón, **nada** en D1 sugiere ni enseña el procedimiento de obtención del éster metílico del ácido (3aR, 4S, 7aR)-4-hidroxi-4-m-toliletinil-octahidro-indol-1-carboxílico (I), (2-cloro-etil)-(3-oxo-ciclohexil)-alquil-éster del ácido carbámico (II), y de enantiómeros de 1-carbalcoxi-4-ceto-perhidro-indol (III), con los reactivos de la invención, sin una etapa de hidrogenación y con los porcentajes de pureza como en la presente invención.

En conclusión, nada en el arte previo proporciona enseñanzas o sugerencias que lleven a la persona medianamente versada en la materia al procedimiento aquí reivindicado, por tal razón, la materia reclamada en la presente invención es inventiva sobre el arte previo citado por el Examinador.

6. Por todo lo anterior, considero que las objeciones presentadas por el Examinador en su primer examen de fondo han sido resueltas y por consiguiente solicito respetuosamente que el Despacho tenga por cumplidos los requisitos de patentabilidad y proceda con la concesión de la patente.

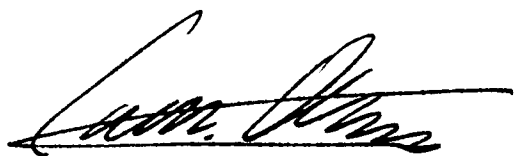
⁵ Manual Andino de Patentes. (2004). Sección 10.2.1 Ejemplo 1 (disponible en <http://www.comunidadandina.org/public/patentes.pdf>).

⁶ Manual Andino de Patentes. (2004). Sección 10.8.1 (disponible en <http://www.comunidadandina.org/public/patentes.pdf>).

OLARTEMOURE

OLARTE MOURE & ASOCIADOS LTDA.

Atentamente,



CARLOS R. OLARTE

C.C. No. 79.782.747 de Bogotá

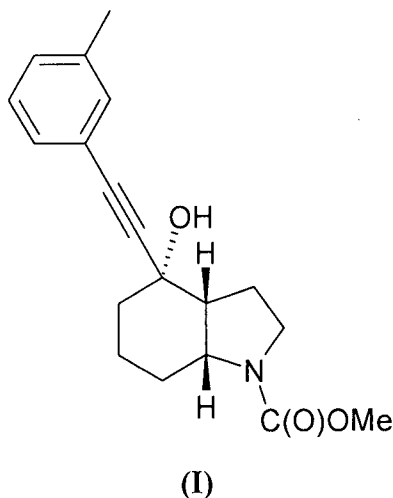
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Anexos:

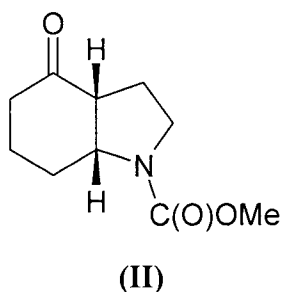
- Capítulo Reivindicatorio Modificado.
- Anexo 1

CAPITULO REIVINDICATORIO MODIFICADO
(Primer Examen de Fondo)

1. Un proceso para la producción de un compuesto de la Fórmula (I), o una sal farmacéuticamente aceptable del mismo:

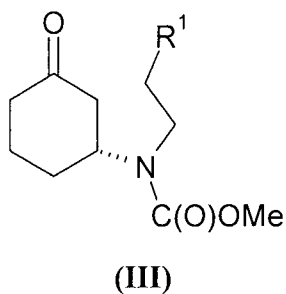


el cual comprende hacer reaccionar 3-etinil-tolueno con un compuesto de la Fórmula (II), o una sal del mismo:



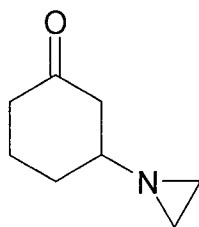
para formar el compuesto de la Fórmula (I), y opcionalmente convertir el compuesto de la Fórmula (I) hasta una sal farmacéuticamente aceptable.

2. Un proceso de acuerdo con la Reivindicación 1, en donde el compuesto de la Fórmula (II) se obtiene mediante la ciclación de un compuesto de la Fórmula (III), o de una sal del mismo:



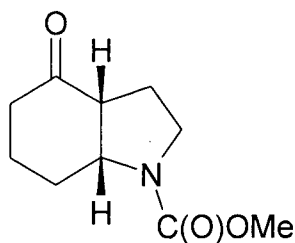
en donde la ciclación se realiza por un solvente orgánico usando una base que comprende una mezcla de pirrolidina y trietilamina; y en donde R^1 es un grupo saliente seleccionado de cloro, bromo o yodo, metanosulfonato o fluorosulfonato.

3. Un proceso de acuerdo con la Reivindicación 2, en donde el compuesto de la Fórmula (III) se obtiene mediante la reacción de un compuesto de la Fórmula $R^1C(O)OMe$ con un compuesto de la Fórmula (IV), o con una sal del mismo:



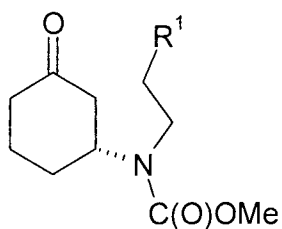
(IV)

4. Un proceso para la producción de un compuesto de la Fórmula (II), o de una sal del mismo:



(II)

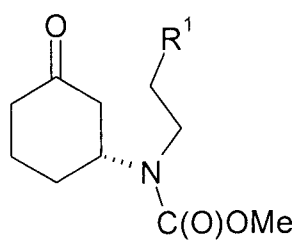
el cual comprende la ciclación de un compuesto de la Fórmula (III), o de una sal del mismo:



(III)

en donde la ciclación se realiza por un solvente orgánico usando una base que comprende una mezcla de pirrolidina y trietilamina; y en donde R^1 es un grupo saliente seleccionado de cloro, bromo, yodo, metanosulfonato o fluorosulfonato.

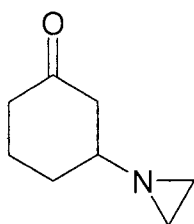
5. Un proceso de acuerdo con la Reivindicación 4, en donde el compuesto de la Fórmula (III) se obtiene de acuerdo con la Reivindicación 3.
6. Un proceso para la producción de un compuesto de la Fórmula (III), o de una sal del mismo:



(III)

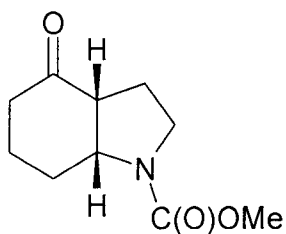
en donde R^1 es un grupo saliente seleccionado de cloro, bromo, yodo, metanosulfonato o fluorosulfonato;

el cual comprende hacer reaccionar un compuesto de la Fórmula $R^1C(O)OMe$ con un compuesto de la Fórmula (IV), o con una sal del mismo:



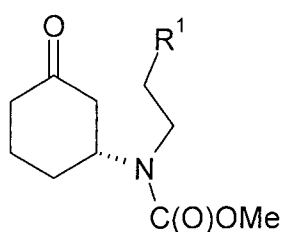
(IV)

7. Un compuesto de la Fórmula (II), o una sal del mismo:



(II)

8. Un compuesto de la Fórmula (III), o una sal del mismo:



(III)

en donde R^1 es un grupo saliente, seleccionado de cloro, bromo, yodo, metanosulfato o fluorosulfonato.

Marco E. Bianchi
Angelo A. Manfredi

High-mobility group box 1 (HMGB1) protein at the crossroads between innate and adaptive immunity

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Summary: Tissue damage occurs often in the life of mammals and is usually repaired. Dying cells are swiftly phagocytosed, but before disappearing, they alert surrounding cells to activate homeostatic programs. They release signals that recruit inflammatory cells to the site of injury, promote cell migration and cell division to replace dead cells, and activate the immune system in anticipation of microbial invasion. Many of these events involve high-mobility group box 1 protein (HMGB1), a nuclear protein that is released passively when necrotic cells lose the integrity of their membranes. HMGB1 behaves as a trigger of inflammation, attracting inflammatory cells, and of tissue repair, recruiting stem cells and promoting their proliferation. Moreover, HMGB1 activates dendritic cells (DCs) and promotes their functional maturation and their response to lymph node chemokines. Activated leukocytes actively secrete HMGB1 in the microenvironment. Thus, HMGB1 acts in an autocrine/paracrine fashion and sustains long-term repair and defense programs. DCs secrete HMGB1 several hours after contact with the first maturation stimulus; HMGB1 secretion is critical for their ability to reach the lymph nodes, to sustain the proliferation of antigen-specific T cells, to prevent their activation-dependent apoptosis, and to promote their polarization towards a T-helper 1 phenotype. These immune responses will also be directed against self-antigens that DCs process at the time of injury and can lead to autoimmunity.

Keywords: cell death, alarmins, cross-presentation, dendritic cells, autoimmunity

Near (cell)-death experiences: signals of necrosis activate repair, immunity, and autoimmunity

Extensive tissue injury as a consequence of mechanical or thermal trauma is not an uncommon event. Just as localized tissue damage gives rise to localized inflammation, massive tissue injury can give rise to generalized sterile inflammation or systemic inflammatory response syndrome. In the past decade, our understanding of sterile inflammation has progressed significantly. It is now clear that multifunctional alarm signals are generated as a consequence of cell and tissue damage. These signals in turn orchestrate a complex homeostatic response, which comprises crucial and possibly mutually dependent events.

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The first set of events is the activation of a repair program. This program involves the direct modification of the bystander cells, which secrete growth and survival factors, and the expansion of local precursor cells. It also involves the attraction and recruitment of inflammatory leukocytes and circulating stem cells. Resident and newly recruited cells secrete cytokines and growth factors and form granulation tissue; eventually, *restitutio ad integrum* also requires the organization of novel extracellular matrix, via deposition by activated fibroblasts and degradation by infiltrating leukocytes.

A second set of events is the activation of a defense program. Microbes are likely to exploit the disruption of the physical (such as the epithelia) and chemical barriers (such as mucoid/protein secretions) of the host caused by trauma. Bystander cells and inflammatory leukocytes activate mechanisms of both passive and active resistance to infection, some of which are preventive, i.e. they occur before the infection itself.

These functional outcomes do not substantially differ from those elicited by the direct recognition of the molecular structures shared by pathogens, referred to as pathogen-associated molecular patterns (PAMPs). PAMP recognition promotes the production of pro-inflammatory cytokines and chemokines and the expression of costimulatory molecules. These factors are essential for induction and regulation of the adaptive immune response to foreign antigens. The study of sterile inflammation clearly indicates that endogenous triggers are equally effective at activating a reparative and protective response, including the activation of the immune system.

In this review, we discuss the role played by high-mobility group box 1 protein (HMGB1) as a signal of tissue damage and in particular in activating defense programs. HMGB1 has special significance, because it was the first molecule convincingly implicated in sterile inflammation, although it can also be involved in non-sterile inflammation. We also discuss its potential roles in autoimmunity. The state of alert of the immune system, induced by dying cells regardless of the actual presence of invading pathogens, is likely to be evolutionary advantageous (1), but it allows for immune responses to be initiated against the antigens that local antigen-presenting cells (APCs) are processing at the time of the injury. This event can lead to autoimmunity, because such antigens are self-antigens. Several excellent reviews extensively cover the evidence linking cell death to persisting autoimmunity (2–4), and we do not discuss this issue further here.

How to recognize an alert signal

Substantial effort has been put forth to identify the signals from injured tissues that trigger the homeostatic responses that

promote repair and activate the immune system. These signals, collectively referred to as damage-associated molecular patterns (DAMPs) or alarmins, possibly comprise quite heterogeneous molecules that share some functional characteristics (5). They should be preferentially released during unscheduled cell death (necrosis) but segregated inside cells dying via apoptosis. They should promote tissue regeneration, and they should directly or indirectly promote acquired T-cell-dependent immune responses. Moreover, viable activated innate immune cells, including dendritic cells (DCs), apparently release these molecules, possibly via non-classical leaderless secretion. It is also tempting to speculate that DAMPs/alarmins would play a crucial role in determining whether antigens from apoptotic cells are cross-presented or not by DCs that are present in or are attracted to injured tissues.

We are particularly interested in a protein that fulfils all these criteria: HMGB1. HMGB1 mostly resides in the nucleus, where it bends DNA and promotes protein assembly on specific DNA targets (6). Our interest was aroused by the differential behavior of HMGB1 in cells undergoing necrosis and apoptosis (7). Necrotic or damaged cells passively release HMGB1 in their environment, whereas in apoptotic cells, HMGB1 binds tightly to chromatin remnants. HMGB1 had previously been recognized as a pro-inflammatory molecule secreted by monocytes and macrophages in response to tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), or lipopolysaccharide (LPS) (8). Thus, HMGB1 might be one of the long-sought molecules that alert the organism to sterile tissue damage. In fact, the supernatant of necrotic cells containing HMGB1 activated bone marrow-derived cells to secrete TNF- α , whereas the supernatant of *Hmgb1*^{-/-} cells was much less effective. Massive liver necrosis induced by paracetaminophen overdose attracted inflammatory leukocytes into the liver, but this influx was significantly reduced by anti-HMGB1 antibodies (7).

HMGB1, an ancient protein

HMGB1 was discovered three decades ago as a nuclear protein that migrates quickly during electrophoresis, and it was named after this property (9). Its gene codes for a single 215 amino acid protein, although the initial methionine is not present in the protein purified from mammalian cells. Despite having a molecular weight of approximately 25 kDa, it migrates at a position of 30 kDa in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels, possibly because of the unusual number of positively charged amino acids it contains.

HMGB1 consists of two fairly rigid, L-shaped DNA-binding domains, each referred to as an 'HMG box' (10), and an unstructured tail that ends with 30 consecutive negatively charged amino acids (Fig. 1). The tail interacts with the HMG boxes and may modulate their intermolecular interactions (11, 12). The HMG boxes are similar in conformation to each other and to other HMG boxes present in numerous other proteins, despite a limited (20%) amino acid identity.

The sequences and structures of HMGB1-related proteins have been strictly conserved during evolution, and genes orthologous or paralogous to human HMGB1 are easily recognizable in all multi-cellular animals (13). Mammals have at least three genes, HMGB1, HMGB2, and HMGB3, which express similar (> 80% identity) but only partially redundant proteins. HMGB1 proteins from all mammals are virtually identical (> 99%), implying that each single residue is under strong selective pressure; HMGB2 and HMGB3 are also strongly conserved.

HMGB1 protein is essential to life, as *Hmgb1* knockout mice die shortly after birth (14). Phenotypic features include small size, ruffled and disorganized fur, long hind paws, and absence of fat. *Hmgb2* and *Hmgb3* knockout mice have very slight phenotypes (15, 16).

HMGB1 is fairly ubiquitous in mammals and is generally abundant (more than 1 million molecules/cell), although some cells are relatively HMGB1-poor (17). HMGB2 and HMGB3 are widely expressed in embryos but have a much more restricted expression pattern in the adult (bone marrow, lymphoid tissues, and testes). HMGB1 is almost always present in the nucleus, although under specific circumstances, some cells contain it in the cytoplasm, on the external side of their plasma membranes, or in extracellular fluids (18).

In the nucleus, HMGB1 binds without sequence specificity to the minor groove of DNA and induces bends in the helical structure (19). This structural change facilitates multiple physical interactions between DNA and various proteins,

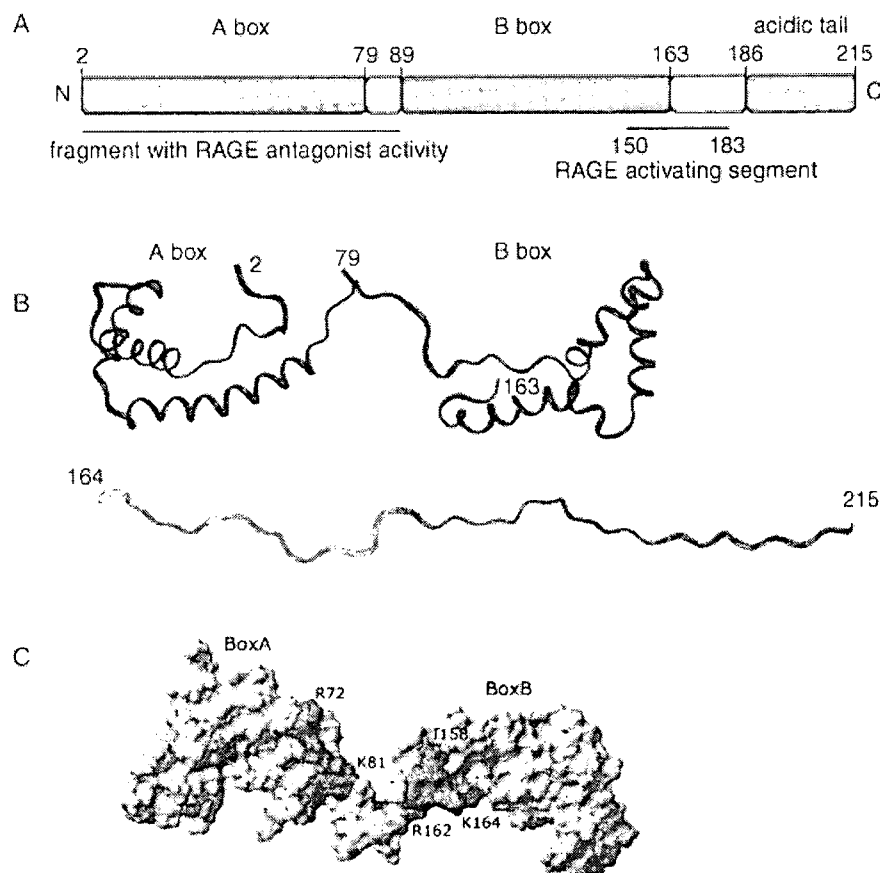


Fig. 1. Structure of HMGB1. (A) HMGB1 is a 25 kDa nuclear protein of 215 amino acids. It comprises two DNA-binding domains (red), boxes A and B, and a negatively charged C-terminal tail (green). The blue boxes are connecting segments. The region required for the activation of RAGE is located between residues 150 and 183. Box A, when isolated from the rest of the molecule, acts as a specific antagonist of HMGB1. (B) Ribbon representation of the boxes and of the unstructured tail. Note the extreme length of the tail when extended. (C) Space-filling representation of the boxes. The two boxes are rotationally free but interact with the acidic tail via the basic residues highlighted in blue. HMGB1, high-mobility group box 1; RAGE, receptor for advanced glycation end products.

including p53, nuclear factor- κ B (NF- κ B), homeobox-containing proteins, recombination activating gene 1/2 (RAG1/2) proteins, and steroid hormone receptors. HMGB1 is an extremely mobile protein. The entire pool of HMGB1 roams the nucleus, resting on a specific DNA site for only fractions of a second. The transient binding to chromatin enables HMGB1 to perform its activities in transcription and other nuclear transactions.

HMGB1 as a late mediator in sepsis

The interest in HMGB1 was rekindled by the realization that the protein has a second function. In the search for a late mediator in the development of sepsis, the Tracey group (8) identified HMGB1 as a protein secreted from monocytes and macrophages following stimulation with LPS, TNF- α , or IL-1 β (Fig. 2). In contrast to TNF- α and IL-1 β , which are secreted within minutes of LPS stimulation, HMGB1 is secreted after several hours, both *in vitro* and *in vivo*. The most striking finding was that neutralizing anti-HMGB1 antibodies protect against LPS-mediated lethality. These data were further supported by results obtained in a mouse cecal ligation and puncture (CLP) model. CLP involves ligation of the cecum followed by the perforation of the cecal stump with a needle, causing intra-abdominal infection (peritonitis) and sepsis. Specific inhibition of HMGB1, using antibodies or recombinant box A, reduced

lethality, even when started 24 h after induction of peritonitis (20). No other treatment is effective so late: antibodies against the macrophage migration inhibitory factor are effective up to 8 h after cecal perforation, and anti-TNF- α antibodies actually increase mortality (21).

These results, although striking, beg the question of how HMGB1 impacts the development of endotoxemia. An early suggestion was that HMGB1 causes inflammatory cells to secrete TNF, IL-1 α , IL-1 β , IL-1RA, IL-6, IL-8, macrophage inflammatory protein (MIP)-1 α , and MIP-1 β , but not IL-10 or IL-12 (22). Thus, HMGB1 might participate in a 'cytokine storm.' However, several recent studies have found that carefully purified HMGB1 is a chemoattractant for a number of cell types, including all inflammatory cells, but causes little secretion of any cytokine from mouse or human macrophages (23–25, L. Cassetta, M. E. Bianchi, and G. Poli, unpublished data). However, supernatants of necrotic cells containing HMGB1 are effective in inducing cytokine secretion, suggesting that the activity is due to the combined action of HMGB1 with another DAMP or a PAMP, which of course would be abundant in a CLP model. Moreover, HMGB1 affects the permeability of endothelia and alveolar and intestinal epithelia (26–28). It might affect sepsis-induced lethality by abrogating the barrier function of epithelia, which keeps bodily fluids separated.

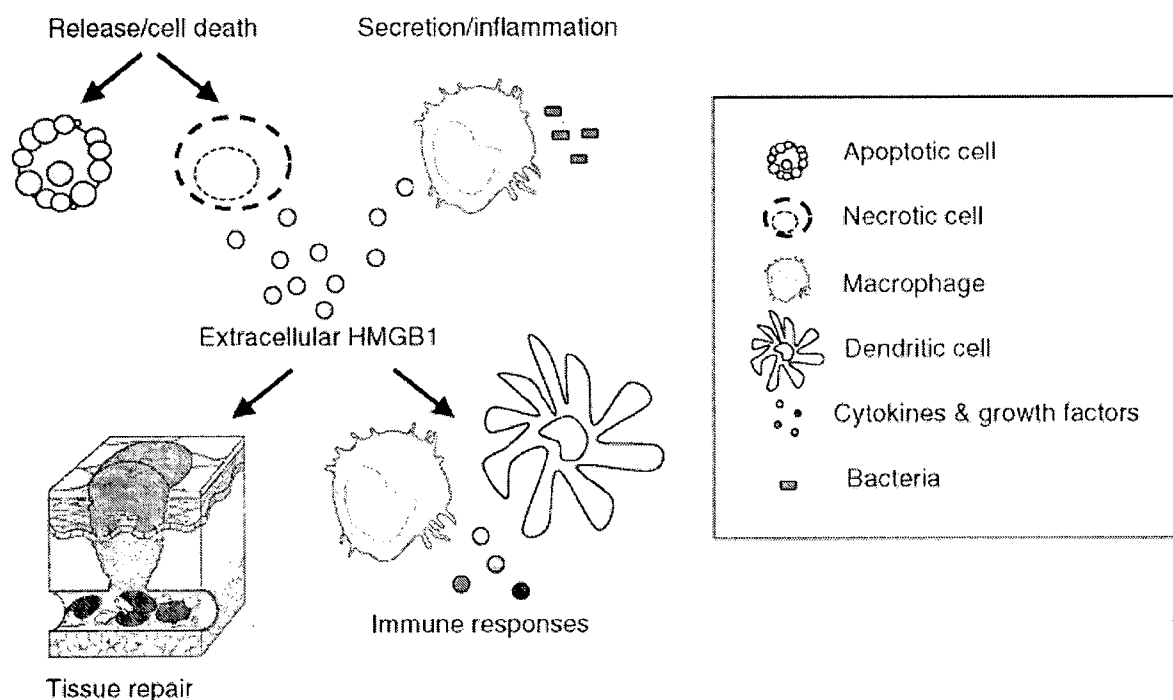


Fig. 2. HMGB1 is released passively by necrotic cells, retained by apoptotic cells, and secreted actively by inflammatory cells. Cells killed by CTLs also release HMGB1. Extracellular HMGB1 initiates tissue repair programs and defense programs that include activation of adaptive immunity. HMGB1, high-mobility group box 1; CTLs, cytotoxic T lymphocytes.

Several flavors of HMGB1: plain, acetyl, phosphoryl, and methyl

- The source of HMGB1, necrotic cells or inflammatory cells (Fig. 2), is of some importance. At the time of death, necrotic cells release HMGB1 either unmodified or acetylated on lysines at positions 2 and 11. HMGB1 secreted by monocytes (that do not die in the process) is heavily acetylated on lysines throughout the length of the protein, creating a potentially huge ensemble of molecular species where acetylation is present or not on specific HMGB1 lysines (29). Acetylation is directed particularly to segments 27–43 and 178–184, which act as nuclear localization signals (NLSs). Neutralization of the positive charges of the NLSs impedes the nuclear import of HMGB1, which becomes relocated to the cytoplasm. In the case of myeloid cells, HMGB1 then is concentrated within a subpopulation of 'secretory' lysosomes, whose contents are discharged into the extracellular medium when cells receive specific signals (30). Several cell types (neurons, smooth muscle cells, and enterocytes) can secrete HMGB1 without dying, although they do not appear to contain secretory lysosomes. The passage of HMGB1 from the cytoplasm across a cellular membrane, be it the lysosomal membrane or the plasma membrane, may be mediated by one or more transporters. Acetylation of HMGB1 appears to be important for the relocation of the protein from the nucleus to the cytoplasm, and it is unclear whether it is also necessary for the passage through lysosomal and/or plasma membranes. Indeed, a second pathway of HMGB1 nuclear/cytoplasmic shuttling exists, involving phosphorylation (31). Thus, non-acetylated HMGB1 may also become available for secretion; secreted HMGB1 however is not phosphorylated. Conversely, lysis of a cell where HMGB1 has been relocated from the nucleus to the cytoplasm, but not yet actively secreted, might lead to the passive release of acetylated HMGB1. Finally, neutrophils mono-methylate HMGB1 on lysine 42 and relocate it to the cytoplasm; secretion of methylated HMGB1 then occurs (32).

Current evidence links directly HMGB1 post-translational modifications to its cytoplasmic relocation and indirectly to its secretion. The molecular difference between variably modified HMGB1 molecules may lead to variable potency in the activation of specific HMGB1 receptors, although this possibility has not been specifically tested.

HMGB1, tissue damage, and receptor for advanced glycation end products

Several instances where HMGB1 is released by damaged tissues or organs are well documented. Importantly, the damage can be

caused not only by trauma but also by the immune system itself: cells killed by cytotoxic T lymphocytes (CTLs) release HMGB1 (33).

Damaged pancreatic β -cells release HMGB1 (34), and indirect clinical evidence indicates that HMGB1 is also released following ventilator-induced lung injury (35). During the acute rejection of heart allografts, there is a significant upregulation of the expression of HMGB1 in the transplanted organ, accompanied by focal necrosis and by the infiltration of inflammatory cells. Blockade of HMGB1 with the functional inhibitor box A reduced the production of inflammatory cytokines and ameliorated the clinical outcome (36).

Acute liver injury, such as following ischemia and reperfusion of the organ, causes the rapid release of HMGB1, which triggers inflammation (37, 38). Likewise, following hepatocyte killing by hepatitis B virus (HBV)-specific CTLs in a mouse model of hepatitis, HMGB1 directs the intrahepatic recruitment of neutrophils and of all other non-antigen-specific inflammatory cells [natural killer (NK), NKT, T, and B cells, monocytes, macrophages, DCs] (39).

As indicated by the examples above, HMGB1's role as chemoattractant for inflammatory cells is undisputed, and the receptor involved in this activity appears to be the receptor for advanced glycation end products (RAGE) (40). RAGE is also a receptor for some S100 proteins, for β -fibrils involved in Alzheimer's disease, and for advanced glycation end products (41). RAGE is expressed at a high level only in lung type-I pneumocytes (42), but it is expressed at a low level in many or all cell types, including neurons, endothelia, and inflammatory cells. RAGE belongs to the immunoglobulin (Ig) superfamily and has three extracellular Ig domains, a single transmembrane segment, and a short intracellular segment that does not contain tyrosines and has no homology to any known protein domain. No direct interactor of the intracellular segment of RAGE is known, but truncation of this segment generates a RAGE with dominant-negative properties. A soluble form of RAGE is also known. HMGB1 binding to RAGE results in the activation of mitogen-activated protein kinases and NF- κ B, although the individual steps in this pathway are not known. No definitive data exist on whether RAGE signals as a multimer (as the existence of a dominant-negative mutant would suggest) or on which surface it interacts with HMGB1. We do know that a fragment of HMGB1, box A, behaves as a competitive inhibitor of HMGB1 (20, 39), implying that box A can bind RAGE without activating it. Indeed, the productive interaction between HMGB1 and RAGE requires the basic stretch between box B and the acidic tail; antibodies directed against this segment neutralize HMGB1 as a RAGE ligand (43).

Some insights on the mechanisms involved in HMGB1-dependent neutrophil recruitment have been obtained recently. HMGB1 promotes the interaction of RAGE with the Mac-1 $\beta 2$ integrin, and antibodies against Mac-1 can block RAGE-dependent activities (44). These findings suggest that RAGE might behave more as an adapter than as a receptor; however, Mac-1 is restricted to neutrophils and macrophages, suggesting that RAGE might need other interactors in other cell types. Interestingly, pertussis toxin blocks HMGB1-elicited migration of smooth muscle cells and DCs (45, 46), suggesting that RAGE might interact with a G-protein-coupled receptor.

RAGE knockout mice are viable and are less susceptible to sepsis than wildtype mice (47). We have recently derived *Rage*^{-/-} mouse embryonic fibroblasts and have verified that their chemotactic response to HMGB1 is severely reduced but not eliminated (A. Raucci and M. E. Bianchi, unpublished data). This finding raises the possibility that RAGE is not the only receptor involved in the chemotactic and tissue repair activities of HMGB1, which would also explain the relatively limited phenotype of *Rage*^{-/-} mice.

HMGB1 and tissue regeneration

HMGB1 also fulfils the prediction that a signal from damaged tissues should promote their regeneration (Fig. 2). *In vitro* in the absence of serum, HMGB1 acts as a mitogen for several cell types; moreover, it also attracts and activates stem cells. HMGB1-loaded beads implanted in normal, healthy mouse muscles (mimicking the passive release from damaged muscle) attract mesoangioblasts injected in the general circulation (43). HMGB1 promotes mesoangioblast colonization of dystrophic muscles (48). HMGB1 also promotes heart regeneration after infarction (49): the injection of HMGB1 into the peri-infarcted left ventricle induced the proliferation and differentiation of new cardiomyocytes, presumably deriving from resident cardiac stem cells. The novel myocytes were functional, and the hemodynamic parameters of the HMGB1-treated hearts were significantly improved.

HMGB1 also has angiogenetic activities, which are obviously required for the restoration of damaged tissues. It promotes the migration and the growth of endothelial cells of various origins, including endothelial progenitor cells, and their organization in tubules (50–52). HMGB1's ability to promote tissue growth and remodeling might also be involved in some of its less benign activities. HMGB1 has been linked to atherosclerosis progression (53–55). HMGB1 is found in atherosclerotic plaques, both released by necrotic cells and

secreted by macrophages and foam cells. Surprisingly, smooth muscle cells, which normally contain little HMGB1, produce a high level of HMGB1 and secrete it, when activated by inflammatory mediators or just by cholesterol. Moreover, in an autocrine/paracrine response to HMGB1, smooth muscle cells proliferate, migrate, and secrete more HMGB1, possibly leading to vessel wall thickening (stenosis) (51). Thus, in response to damage or inflammation, tissue growth and remodeling could actually lead to an exacerbation of a pathogenetic process. A potentially similar scenario might also be relevant for rheumatoid arthritis, the other degenerative disease where HMGB1 appears to play a role (56, 57).

All of HMGB1's activities in tissue regeneration involve RAGE, as anti-RAGE antibodies or dominant-negative RAGE interfere with them. However, it has not been tested whether *Rage*^{-/-} mice are defective in tissue repair.

HMGB1 and Toll-like receptors

The moderate phenotype of RAGE knockout mice suggest that other HMGB1 receptors exist, and the ability of HMGB1 to act as a DAMP suggests that Toll-like receptors (TLRs) might be involved. Candidates are TLR2 and TLR4: murine macrophage cell lines transfected with dominant-negative constructs of TLR2 and TLR4 show a decreased activation on stimulation with HMGB1 (58). Significantly, hepatic ischemia/reperfusion damage, which is mediated by HMGB1 (37), involves TLR4 signaling (59). However, primary macrophages from wildtype, TLR4 knockout, and TLR2 knockout mice respond similarly to HMGB1 (60). These conflicting results might be due to a partial overlap in RAGE and TLR2/4 pathways or to subtle differences in the nature of HMGB1 interacting with the receptors (plain versus post-translationally modified, alone or in combination with other DAMPs or PAMPs).

HMGB1 has been shown recently to interact with TLR9 and to enhance the TLR9-dependent immunostimulatory effect of CpG DNAs on macrophages, DCs, and B cells (24, 25); however, some reports are inconsistent with these findings (61). HMGB1 binds CpG-containing single-stranded DNA preferentially, although the recognition might depend more on structure than on sequence, and enhances responses triggered by CpG DNA (secretion of IL-6, IL-12, IFN- α , and TNF- α). Both extracellular and intracellular HMGB1 (originating from the cell nucleus) are recruited to vesicles that contain TLR9, establishing a physical interaction with it (although the binding surfaces have not been identified) and apparently accelerate the formation of the CpG-DNA-TLR9 complex. HMGB1 does not affect the uptake of CpG DNA or

its entry into early endosomes but rather accelerates the redistribution of TLR9 from the endoplasmic reticulum to the early endosomes in response to CpG DNA. Loss of HMGB1 leads to a defect in the response to CpG DNA in terms of IL-6, IL-12, TNF- α , and inducible nitric-oxide synthase expression.

The lack of intracellular TLR9-associated HMGB1 can be compensated for by extracellular HMGB1, indicating that HMGB1 can shuttle in and out of immune cells. Most interestingly, RAGE is also involved in this process, since anti-RAGE antibodies reduce CpG-mediated responses and as *Rage*^{-/-} cells respond poorly to CpG DNA. The process is HMGB1 specific, because ligation of RAGE by S100 does not affect CpG-dependent responses. Given the mechanism of HMGB1 interaction with TLR9, one can expect that recognition of PAMPs by other intracellular TLRs might be affected by HMGB1 and RAGE as well. Overall, the interaction of HMGB1 with multiple TLRs makes it a crucial protein in innate immunity.

HMGB1 and adaptive immunity

The belated recognition of HMGB1 as an extracellular signal prompted us to investigate whether HMGB1 had any effect on T-cell-dependent acquired immune responses against nominal antigens. Indeed, we found that HMGB1 behaves as an endogenous immune adjuvant (62).

HMGB1 elicited a striking increase in the titers of high-affinity hypermutated IgG when co-injected with the soluble nominal antigen ovalbumin. Moreover, it was sufficient to reconstitute the immunogenicity of apoptotic 'self' cells *in vivo*. We injected mice with syngeneic apoptotic lymphoma cells, which are antigenic but poorly immunogenic (63). After 14 days, we challenged them with living lymphoma cells and evaluated the tumor development. As expected, all injected mice subsequently challenged with living lymphoma cells developed the tumor. In contrast, mice injected with apoptotic lymphoma cells in the presence of necrotic *Hmgb1*^{+/-} fibroblasts rejected the tumor. They also rejected the tumor after 2 months of further challenge with living lymphoma cells, indicating that they developed a memory response. The adjuvant effect of necrotic cell supernatants abated when fibroblasts were derived from *Hmgb1*^{-/-} fibroblasts or when functional inhibitors of extracellular HMGB1 were used (64). Interestingly, recombinant HMGB1 made in bacteria was less active as an adjuvant than HMGB1 present as a component of necrotic cells (A. Manfredi, unpublished results), raising the possibility that acetylated HMGB1 might be more potent in this context than non-acetylated HMGB1.

The effects of HMGB1 on acquired immunity involve DCs

Adjuvants work through several mechanisms. They facilitate the trafficking of inflammatory leukocytes at the site of antigen injection; they promote the activation of APCs and of DCs, in particular, facilitating their migration to the lymph nodes and enhancing their ability to stimulate resting antigen-specific T cells. In some cases, adjuvants induce the release of cytokines that influence indirectly other immune cells.

HMGB1 facilitates the trafficking of inflammatory leukocytes, but this mechanism does not preclude the existence of other effects. We, therefore, investigated whether HMGB1 was involved in the maturation of DCs in response to necrotic cells (62). In fact, supernatants from necrotic HeLa cells containing HMGB1 activated DCs (Fig. 3); the addition of HMGB1-specific antibodies or of the HMGB1 inhibitory fragment box A to the supernatants abrogated DC maturation. Moreover, the supernatants from *Hmgb1*^{-/-} necrotic cells had a much reduced ability to activate and induce maturation of DCs in comparison with the supernatants from wildtype cells. Similar results were obtained by the Tracey group (64), who also found that HMGB1-activated DCs induced the proliferation and the clonal expansion of alloreactive T cells, with strong T-helper 1 cell (Th1) polarization.

Immature DCs are CCL5-sensitive, whereas mature DCs switch to CCL21 sensitivity and have an improved ability to migrate to secondary lymph nodes (65). This migration is a crucial event, because DCs usually acquire foreign antigens in peripheral tissues, while they present them to naive T cells after several hours and in the T-cell zone of the draining lymph nodes. The mobility of DCs and their trafficking into and through lymphatic vessels to lymph nodes are therefore critical for their function. Yang *et al.* (46) showed that HMGB1 switches DC chemokine responsiveness from CCL5 sensitivity to CCL21 sensitivity. Moreover, HMGB1 itself is a chemoattractant for immature but not mature DCs.

These data indicate that the adjuvant properties of HMGB1 proceed via DC activation, maturation, and mobilization, allowing their productive interaction with T cells. However, there is another unexpected twist to this story: immature DCs secrete their own HMGB1 when activated (Fig. 3). Several independent stimuli induce HMGB1 secretion, for example LPS, TNF- α , and CD40L (66, 67, I. E. Dumitriu, A. Manfredi, M. E. Bianchi, P. Rovere-Querini, unpublished results), suggesting that HMGB1 secretion is a general feature of maturing DCs. Importantly, HMGB1 secretion appears essential for DC functions, because DCs matured in the

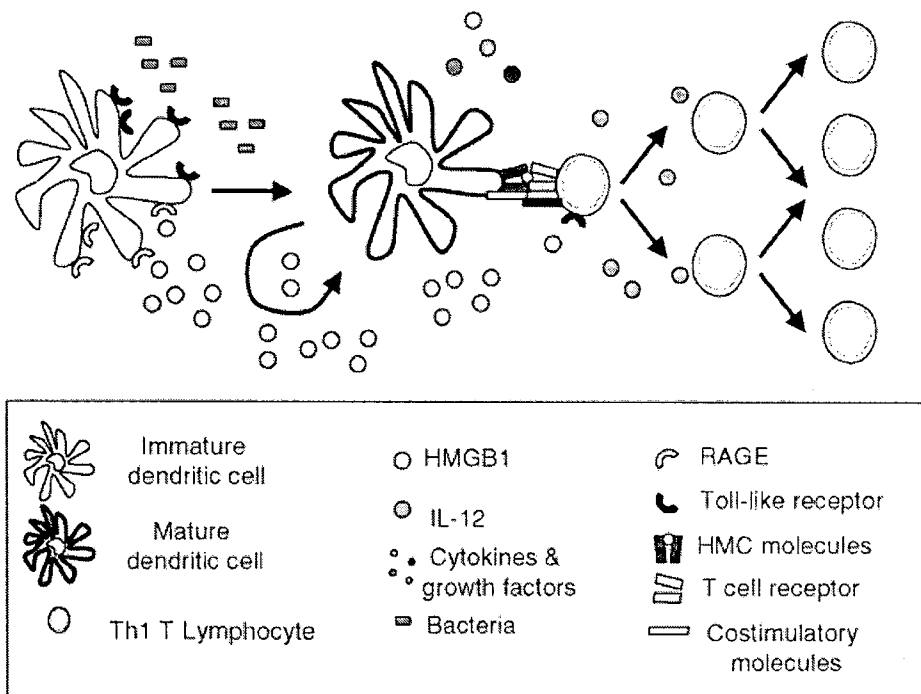


Fig. 3. Immature DCs can be activated by a number of different stimuli, including extracellular HMGB1 binding to its receptors RAGE and possibly TLR9, TLR2, and TLR4. As a result of activation, DCs secrete HMGB1, which sustains their activation, induces their maturation, and promotes their migration to lymph nodes, where they encounter naive T cells. HMGB1 also acts directly on T cells, promoting their proliferation, their resistance to activation-induced apoptosis, and their differentiation toward a Th1 phenotype. DCs, dendritic cells; RAGE, receptor for advanced glycation end products; HMGB1, high-mobility group box 1; TLR, Toll-like receptor; Th1, T-helper 1 cell.

presence of anti-HMGB1 antibodies, the competitor truncated molecule box A, or anti-RAGE antibodies fail to upregulate the CD80, CD83, and CD86 surface markers, to produce IL-12, and to promote the clonal expansion and the survival of naive T cells (66, 67). RAGE is critically involved in this autocrine/paracrine circuit, because its blockade *in vitro* reproduces the effects of the blockade of HMGB1, and DCs from *Rage*^{-/-} mice are functionally defective *in vitro* and *in vivo* (46, 66, 67, Authors' unpublished results).

DCs that mature upon TLR4 activation by LPS phosphorylate two members of the mitogen-activated protein kinase family (p38 and ERK1/2) and activate the canonical inhibitor of NF- κ B (I κ B) kinase (IKK β), with phosphorylation and subsequent degradation of I κ B α . As a result, NF- κ B translocates into the nucleus. In the presence of HMGB1 inhibitors, NF- κ B is activated, but activation subsides quickly. This result together with the fact that exposure of DCs to HMGB1 also activates NF- κ B suggested that the active release of HMGB1 is required to sustain, in an autocrine/paracrine fashion, NF- κ B long-term activation and the maturation of DCs once initiated (46, 66, 67). This requirement should be particularly relevant for these highly mobile cells, which acquire antigens in peripheral tissues, possibly in the presence of microbial signals, migrate

through lymphatic vessels, and present antigens to T cells in lymphoid organs.

Beyond DCs, on to T cells

Released HMGB1 not only enhances the ability of DCs to productively activate T cells but also acts directly on T cells (66) (Fig. 3). Naive CD4⁺ T cells, stimulated by direct cross-linking of the CD3 and CD28 membrane molecules, in the presence of DC-conditioned medium containing HMGB1 polarize toward a Th1 phenotype and also resist activation-induced apoptosis. These effects are abrogated by HMGB1 inhibitors. Moreover, bacterially produced HMGB1 could not replace the protein secreted by DCs, further suggesting that post-translational modifications, including acetylation, are important for adaptive immune responses.

Apoptosis has its advantages

When cells have the time to program their own death, phagocytosis is their destiny. Phagocytosis is not only a waste disposal program but also crucial to promote the resolution of acute inflammation, because the recognition of apoptotic cells actively 'reprograms' the phagocyte to suppress the release of

pro-inflammatory mediators and promote the secretion of immunosuppressive cytokines, such as IL-10 and particularly transforming growth factor- β 1 (TGF- β). Phagocytes must directly contact apoptotic cells; after the contact, they become licensed to respond in an autocrine/paracrine manner to the cytokines they secrete (68). Licensed macrophages also secrete growth and survival factors, with ensuing expansion of local precursor cells and replenishment of differentiated cells (69). Vascular endothelial growth factor (VEGF) is one of these secreted molecules, which also inhibits spontaneous and ultraviolet irradiation-induced apoptosis in surrounding endothelial and epithelial cells (70).

Apoptosis is also important for the development of acquired immune responses, based on the random generation of antigen receptors in T and B lymphocytes. One issue is particularly relevant here: cross-presentation, or the ability of DCs, and in some studies B lymphocytes and macrophages, to process antigens contained in the apoptotic cells they phagocytose. Cross-presentation is possibly important in several settings, including the initiation of immunity against pathogens that do not infect APCs. Antigens acquired from phagocytosed cells can be funneled into the major histocompatibility complex (MHC) class I pathway, thus effectively activating antigen-specific CD8⁺ T cells, and into the MHC class II pathway, thus presenting antigens from apoptotic cells to CD4⁺ T cells. In the presence of TLR-activating stimuli, phagosomes containing apoptotic cells mature with enhanced kinetics and preferentially yield epitopes that associate with nascent class II molecules and thus reach the plasma membrane (71).

Self-antigens inevitably will be cross-presented together with non-self antigens that are possibly present in apoptotic cells. The outcome of cross-presentation *in vivo* is variable: some studies demonstrate the establishment of tolerance and others the activation of immune responses. At 2 weeks of age, in several mouse strains, a physiological wave of β -cell death occurs in pancreatic islets. In non-obese diabetic (NOD) mice, which are a model of type 1 diabetes, such physiological β -cell death precipitates cross-presentation by DCs, which in turn promotes disease and eventually leads to destructive insulinitis (72). The exposure of islet antigens would result in the expansion of autoreactive T-cell clones in the draining lymph nodes, which evaded clonal deletion (73). However, the same pathway, which is pathological in NOD mice, can result in the establishment of peripheral tolerance to the same antigens in other mouse strains. These results indicate that apoptotic cells represent a potential reservoir of antigens, which under specific circumstances can lead to tolerance or priming, depending on signals that are associated with the dying cells (74).

How will HMGB1 affect cross-presentation? After all, most phagocytosed apoptotic cells are full of HMGB1, an endogenous adjuvant that can interact with TLRs. Information is sparse, but intriguing. The first question is whether HMGB1 will be cross-presented itself and give rise to auto-antibodies against it. Here, the answer is definitely yes. Anti-HMGB1 antibodies are found in a high proportion of patients with various autoimmune diseases (75), and Th cells autoreactive to HMGB1 can be cloned (76). The second question is whether HMGB1 will elicit or enhance cross-presentation in DCs. The experiment has not been carried out, but one might predict the answer is yes. HMGB1 has been shown to activate TLRs, and TLR activation directs cross-presented antigens to an MHC class II context (71). Finally, will HMGB1 from ingested cells activate cross-presentation in the cells that phagocytosed them? It has been found that macrophages that ingest apoptotic cells start secreting HMGB1 (77). It would be interesting to know whether the secreted HMGB1 originates from the macrophages or is salvaged from the ingested cells. Even more critically, do ingested cells activate macrophages because they contain HMGB1? An experiment where macrophages ingest apoptotic *Hmgb1*^{-/-} cells would be most illuminating.

Based on the data currently available, we expect HMGB1 to promote cross-presentation of self-antigens to T cells. Certainly, when injected with apoptotic cells in a context similar to vaccination, HMGB1 enhances the immunogenicity of apoptotic cells (62). Whether in other contexts tolerance would be favored is an open question.

Systemic lupus erythematosus, or the disadvantages of apoptosis

Is an intriguing model to study the cross-presentation of antigens associated with dying cells, because defective clearance of cell debris, development of anti-nuclear antibodies, and increased bioavailability of type I IFN all represent hallmarks of the disease (4, 78). Anti-chromatin and in particular anti-nucleosome autoantibodies correlate with disease activity and possibly represent the initial autoantibodies in the natural course of the disease.

Nucleosomes are present not only in the blood of SLE patients (79) but also in the blood of healthy individuals, who excrete nucleosome-sized DNA in urine (80, 81). Thus, nucleosomes are poorly immunogenic in normal conditions. Can HMGB1 play a role in making nucleosomes more immunogenic? We have shown that HMGB1 binds tightly to

chromatin during apoptosis and is retained within the remnants of the apoptotic cells, even after they undergo lysis (7). However, the Pisetsky group (82) has noted that late apoptotic cells do release some HMGB1; it has long been known that some nucleosomes are leaked out from late apoptotic cells. We suggest that the quantitatively minor amount of nucleosomes and HMGB1 leaked out from apoptotic cells are bound together in a complex, just as they are in the cell remnants of apoptotic cells, and the nucleosome-HMGB1 complexes might be more immunogenic than 'bare' nucleosomes. The basic idea is that HMGB1 and DNA might activate TLR9 receptors in APCs, thus increasing the immunogenicity of histones that are present in the same complex. In fact, anti-HMGB1 polyclonal antibodies immunoprecipitate histones from supernatants of late apoptotic cells (M. E. Bianchi, unpublished data), and HMGB1 is present in chromatin that activates autoreactive B cells (24). B cells expressing a transgene-encoded AM14 receptor recognize IgG2a and proliferate in response to IgG2a-chromatin complexes in a TLR9-dependent way (83). PL2-3, a monoclonal IgG2a antibody specific for histone H2A/H2B, activates AM14 B cells when bound to chromatin containing HMGB1; however, it does not form complexes that bind to AM14 B cells when mixed with supernatants from *Hmgb1*^{-/-}

cells (24). Clearly, the critical experiment will be to test nucleosomes originating from wildtype and *Hmgb1*^{-/-} apoptotic cells for their ability to elicit anti-histone and anti-DNA autoantibodies in mice and eventually to precipitate a lupus-like condition.

Conclusion

HMGB1 has been shown to be critical for nuclear transactions, and it is now being studied rigorously for its role in tissue repair and innate immunity. Its study in the context of adaptive immunity is still in its infancy, but such studies might lead to significant advances in understanding how the immune system decides what is self and what is non-self.

Note added in proof

A new paper (84) reports that cells dying by apoptosis after X-ray irradiation or treatment with oxaliplatin or anthracyclines secrete HMGB1 after caspase activation. Cells dying by apoptosis induced by other treatments do not secrete HMGB1. The HMGB1 secreted by dying cells promotes antigen cross-presentation by dendritic cells, by interacting with their TLR4 receptor, and thus promotes an antitumor immune response.

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