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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 06-021510- -00008-0000

Fecha: 2010-12-06 15:56:49 Dep. 2020 DIR.NUEVASCR
Tra. 11 PATENTEIYII Eve: 378 FASENACIONALI
Act. 444 RESPUESTAREQUE Folios: 43

P-1938

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

DIVISION DE NUEVAS CREACIONES

E.

S.

D.

Re: Expediente No. **06.021.510**

Solicitud de patente a nombre de **ORTHO-McNEIL – JANSSEN PHARMACEUTICALS, INC**

Yo, DILIA RODRIGUEZ D'ALEMAN, identificada como aparece al pie de mi firma, domiciliada en la carrera 11 No. 86-53, piso 6, obrando como apoderada de la sociedad **ORTHO-McNEIL – JANSSEN PHARMACEUTICALS, INC**, domiciliada en New Jersey, Estados Unidos, solicitante de la patente de la referencia, en respuesta al oficio No. 11683 notificado por fijación en lista el 10 de septiembre de 2010, relacionado con el concepto técnico de fondo No. 2995, me permito manifestar:

1. Con fundamento en la facultad que confiere el artículo 34 de la Decisión 486 al solicitante para modificar la solicitud de patente en cualquier momento del trámite, presento junto con este memorial una nueva descripción y un nuevo capítulo reivindicatorio, el cual consta de 1 a 29 reivindicaciones y reemplaza en su totalidad el capítulo reivindicatorio presentado anteriormente.

En cuanto a estas modificaciones, me permito decir que las mismas no amplían de ninguna manera la materia objeto de la presente solicitud y se realizaron con el ánimo de superar las objeciones de claridad y calidad de la traducción, planteadas por el examinador en su concepto técnico.

2. Claridad de la Solicitud

El examinador objeta la claridad de la descripción, argumentando que no demuestra la acción terapéutica de los compuestos ni datos comparativos, por lo que el efecto técnico de las moléculas descritas no es evidente. Al respecto, el solicitante le recuerda a esa oficina que ni el Artículo 28 ni ninguno otro Artículo de la Decisión 486 le exige al solicitante presentar la información que el examinador esta requiriendo. En cuanto al efecto técnico de las moléculas descritas, se remite al señor examinador a lo divulgado en las páginas 5 – 6 y 41 - 45 de la descripción, donde se establece claramente cual es el efecto técnico de los compuestos de la invención.

Por otro lado, las figuras 1 – 5 claramente muestran los resultados de pruebas comparativas entre los compuestos de la presente solicitud y los del arte previo,

P-1938

Clarke, Modet & Cº

COLOMBIA

2

representado por la anterioridad D1. El técnico con habilidad ordinaria en la materia puede leer dichas figuras y establecer los beneficios de la tecnología de la solicitud.

A pesar de lo anterior, el solicitante adjunta una declaración hecha por el inventor, en la cual se presentan de manera más clara las ventajas de la invención, así como los resultados de pruebas comparativas entre los compuestos de la presente solicitud y los reportados por el arte previo más cercano, como se reportan en las figuras 1 a 3 de la solicitud, la anterioridad D1 citada por esa oficina.

Por último, el examinador objeta la claridad de las reivindicaciones, con el fin de superar dicha objeción el solicitante presenta un nuevo capítulo reivindicatorio en el cual se subsanan todas las objeciones presentadas.

3. Excepciones a la patentabilidad

Con el fin de subsanar las objeciones presentadas a la patentabilidad de la solicitud, el solicitante presenta un nuevo capítulo reivindicatorio donde se eliminan las reivindicaciones de método de tratamiento.

5. Nivel Inventivo

El examinador objeta la altura inventiva de la presente solicitud, argumentando que la anterioridad US 6.251.8664 (en adelante D1) ya ofrecía una solución al problema técnico planteado en la presente solicitud por lo que, según el examinador, las sustituciones de los aminoácidos planteadas por el solicitante ya eran obvias a la luz de D1.

Al respecto, el solicitante quisiera anotar que no se proporciona enseñanza o sugerencia alguna en D1 que le permita a un técnico con habilidad ordinaria en la materia inferir o deducir que cualquier otra sustitución de aminoácidos en la secuencia tendría el mismo efecto terapéutico. Se le recuerda al examinador que las secuencias genéticas no son piezas modulares donde la actividad de la secuencia es independiente de las sustituciones que se realicen. No existe en el estado del arte ni entre los conocimientos básicos del técnico con habilidad ordinaria en la materia, forma alguna de predecir si una sustitución dentro de una secuencia tendrá el efecto deseado o no, por lo que la afirmación del examinador carece de todo sustento técnico.

Sumado a lo anterior, como se puede apreciar en la declaración del inventor que se anexa la presente solicitud se enfoca en un compuesto TPO que tiene un residuo β -(2-naftil)alanina, 2-Nal, y una sarcosina o alanina en el residuo 14 de la

Clarke, Modet & C^o

COLOMBIA

3

secuencia. La descripción es clara al establecer detalladamente las propiedades de los compuestos con el residuo 1-Nal y 2-Nal sustituidos por triptófano, así como los diferentes resultados obtenidos *in vitro* e *in vivo* entre los compuestos que contienen los residuos 1-Nal y 2-Nal. D1 no enseña los compuestos peptídicos como se reclaman.

Como se aclara en la declaración, el residuo 2-Nal proporciona resultados inesperados sobre el residuo 1-Nal que no se podrían predecir de tal sustitución. Estos resultados inesperados diferencian los compuestos de la presente solicitud, (IEGPTLRQ(2-Nal)LAAR-Sar)₂-K-NHz, comparados con el dímero correspondiente pero con 1-Nal como sustituyente. La enseñanza general de sustituir triptófano con naftilalanina para producir péptidos sintéticos de acuerdo con D1 no proporciona ninguna evidencia relacionada con los resultados inesperados.

Como se muestra en la descripción, ver páginas 23 – 25 y figuras 1 – 5, la presente solicitud ofrece resultados inesperados con los compuestos actualmente reclamados. Sin importar los alegatos que sostienen que existe una expectativa de que los compuestos de la presente solicitud tengan la misma actividad del compuesto de D1, que comprende 1-Nal, los solicitantes han demostrado que los compuestos tienen propiedades muy diferentes. Por lo tanto, es claro que los argumentos del examinador no tienen sustento técnico. De hecho, el compuesto de la presente solicitud exhibe una potencia 10 veces menor que el compuesto 1-Nal del arte previo, algo que era completamente inesperado. Esto demuestra que no es posible predecir si compuestos similares con estructuras similares van a tener las mismas propiedades o no.

En la declaración anexa se demuestra, entre otras cosas, lo siguiente:

- Que el compuesto GW39058 de D1, que se muestra en el artículo de De Serres y colaboradores adjunto, denominado en adelante “el dímero PEG 1-Nal” es más potente que el compuesto TPO 1, en otras palabras “el dímero PEG 2-Nal”, en un ensayo de reporte de luciferasa en el cual las células de murina se modifican para expresar de manera estable el receptor TPO humano.
- Que el dímero PEG 1-Nal es más potente que el dímero PEG 2-Nal en un modelo de rata *in vivo* en el cual el conteo de plaquetas se observa en respuesta a la dosis.
- La potencia y la actividad intrínseca inferior del dímero PEG 2-Nal tiene una ventaja potencial en la reducción o prevención de los efectos laterales tras la administración del dímero PEG 2-Nal a

Clarke, Modet & Cº

COLOMBIA

4

humanos.

- Que la potencia y la actividad intrínseca inferior del dímero PEG 2-Nal puede ayudar a determinar la biodisponibilidad del dímero PEG 2-Nal en humanos, lo que es un prerrequisito para la aprobación de un medicamento por parte de la FDA.
- Que el efecto inmediato del dímero PEG 2-Nal en los niveles de plaquetas tras la administración a humanos indica que los humanos son más sensibles al compuesto de lo esperado.
- Que los niveles en plasma del dímero PEG 2-Nal en humanos durante la totalidad del rango de tiempo de medición solo fue detectable a dosis muy altas.
- Que tanto D1 como el artículo de De Serres y colaboradores no proporcionan sugerencia alguna al técnico con habilidad ordinaria en la materia, de que el dímero PEG 2-Nal pudiera ser empleado a dosis tan bajas y aún proporcionara (a) la habilidad de determinar importantes parámetros PK del dímero PEG 2-Nal en humanos y/o (b) una respuesta biológica apropiada en humanos.

En conclusión, la declaración y los demás documentos anexos muestran que los resultados de reemplazar un residuo 1-Nal por un residuo 2-Nal no serían predecibles.

En resumen, me permito decir que gracias a las modificaciones expuestas en el punto 1 de este memorial y los argumentos anteriormente mencionados, la presente solicitud cumple cabalmente con los requisitos de claridad, unidad, patentabilidad y nivel inventivo respecto a los compuestos expuestos en el capítulo reivindicatorio adjunto. Por lo tanto, solicito comedidamente a ese despacho se sirva reconocer estos requisitos y conceder el privilegio de patente de invención a la solicitud en estudio.

Por último, solicitamos a ese despacho pronunciarse en relación con todos los argumentos presentados en este memorial dando estricta aplicación al principio del debido proceso consagrado en el artículo 29 de la Constitución Nacional. De igual forma, si el examinador considera que subsisten razones para objetar la presente invención ruego expedir otro examen de fondo con los debidos motivos y razones que sustenten su objeción con el fin de que mi mandante tenga la posibilidad de exponer sus argumentos con base en la interpretación de los artículos 45 de la Decisión 486 -el cual otorga la facultad al examinador para emitir los exámenes de fondo-, 35 y 59 del Código Contencioso Administrativo

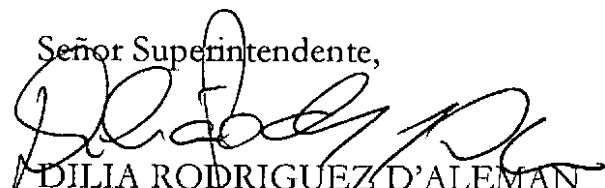
Clarke, Modet & C^o

5

COLOMBIA

que señala que: "habiéndose dado oportunidad a los interesados para expresar sus opiniones, y con base en las pruebas e informes disponibles, se tomará la decisión que será motivada al menos en forma sumaria si afecta a particulares" y "(.) deberá proferirse la decisión definitiva. Esta se motivará en sus aspectos de hecho y de derecho, y en los de conveniencia si es el caso", respectivamente (el subrayado es nuestro).

Señor Superintendente,



DILIA RODRIGUEZ D'ALEMAN

CC. 41.654.882 de Bogotá

TP 20824

Cód. 008

REIVINDICACIONES

1.- Un compuesto peptídico que se une a un receptor de la trombopoyetina, en donde el compuesto peptídico comprende (H-IEGPTLRQ(2-Nal)LAARX₁₀)₂-K(NH₂), en donde X₁₀ se selecciona del grupo que consiste de sarcosina o β-alanina, y en donde 2-Nal es β-(2-naftil)alanina.

2.- El compuesto de la reivindicación 1, caracterizado porque el compuesto peptídico está unido covalentemente a un polímero hidrofílico.

3.- El compuesto de la reivindicación 2, caracterizado porque el polímero hidrofílico tiene un peso molecular promedio de entre 500 a 40,000 daltons.

4.- El compuesto de la reivindicación 2, caracterizado porque el polímero hidrofílico tiene un peso molecular promedio de entre 5,000 y 20,000 daltons.

5.- El compuesto de la reivindicación 2, caracterizado porque el polímero se selecciona del grupo que consiste de polietilenglicol, polipropilenglicol, ácido poliláctico y ácido poliglicólico.

6.- El compuesto de la reivindicación 5, caracterizado porque dicho compuesto está unido de manera covalente a polietilenglicol.

7.- El compuesto de la reivindicación 1, caracterizado porque cada una de las subunidades diméricas del compuesto está unida covalentemente a un polímero hidrofílico.

8.- Una composición farmacéutica que comprende el compuesto de la reivindicación 1, en combinación con un portador farmacéuticamente aceptable.

9.- Una composición polipeptídica fisiológicamente activa, sustancialmente no inmunogénica, soluble en agua, que comprende un compuesto peptídico de la reivindicación 1, acoplado con un agente de acoplamiento a al menos un polímero que tiene un peso molecular de entre 500 y 20,000 daltons, seleccionado del grupo que consiste de polietilenglicol y propilenglicol, en donde el polímero está no sustituido o sustituido con grupos alcoxi o alquilo, los grupos alcoxi o alquilo poseen menos que 5 átomos de carbono.

10.- La composición de la reivindicación 9, caracterizada porque el polímero tiene un peso molecular de 750 a 15,000 daltons.

11.- La composición de la reivindicación 9, caracterizada porque el polímero tiene un peso molecular de 5,000 a 10,000 daltons.

12.- La composición de la reivindicación 9, caracterizada porque el polímero es polietilenglicol.

13.- Una composición polipeptídica sustancialmente no inmunogénica, soluble en agua, que comprende la composición de la reivindicación 9 y un portador farmacéuticamente aceptable.

14.- El compuesto de la reivindicación 1, caracterizado porque dicha secuencia de aminoácidos está ciclizada.

15.- El compuesto de la reivindicación 1, caracterizado porque dicha secuencia de aminoácidos está dimerizada.

16.- El compuesto de la reivindicación 1, caracterizado porque el compuesto comprende la siguiente secuencia de aminoácidos: I E G P T L R Q (2-Nal) L A A R A, y en donde 2-Nal es β -(2-naftil) alanina.

17.- Un compuesto peptídico que se une al receptor de trombopoietina, en donde dicho compuesto peptídico comprende (H-IEGPTLRQ(2-Nal)LAAR(Sar))₂ K-NH₂, en donde 2-Nal es β -(2-naftil) alanina.

18.- El compuesto de la reivindicación 17, caracterizado porque el compuesto está unido covalentemente a un polímero hidrofílico.

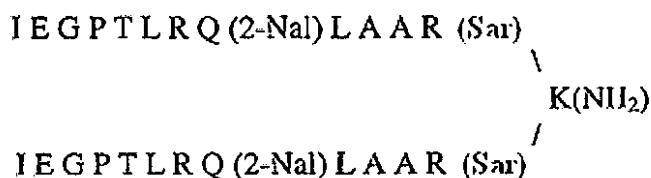
19.- El compuesto de la reivindicación 18, caracterizado porque dicho polímero se selecciona del grupo que consiste de polietilenglicol, polipropilenglicol, ácido poliláctico y ácido poliglicólico.

20.- El compuesto de la reivindicación 19, caracterizado porque dicho compuesto está unido covalentemente a polietilenglicol.

21.- El compuesto de la reivindicación 20, caracterizado porque el polietilenglicol tiene un peso molecular promedio de entre 5,000 y 20,000 daltons.

22.- El compuesto de la reivindicación 21, caracterizado además porque el polietilenglicol se selecciona del grupo que consiste de monometoxipolietilenglicol (MePEG-OH), monometoxipolietilenglicol-succinato (MePEG-S), monometoxipolietilenglicol-succinato de succinimidilo (MePEG-S-NHS), monometoxipolietilenglicol-amina (MePEG-NH₂), monometoxipolietilenglicol-tresilato (MePEG-TRES) y monometoxipolietilenglicol-imidazolil-carbonilo (MePEG-IM).

23.- Un compuesto peptídico que se une al receptor de trombopoietina que tiene la siguiente fórmula:



en donde (2-Nal) es β -(2-naftil)alanina y (Sar) es sarcosina.

24.- El compuesto de la reivindicación 23, caracterizado porque el compuesto esta unido covalentemente a un polímero hidrofílico.

25.- El compuesto de la reivindicación 24, caracterizado porque el polímero hidrofílico se selecciona del grupo que consiste de polietilenglicol, polipropilenglicol, ácido poliláctico y ácido poliglicólico.

26.- El compuesto de la reivindicación 24, caracterizado porque el polímero hidrofílico es polietilenglicol.

27.- El compuesto de la reivindicación 26, caracterizado porque el polietilenglicol tiene un peso molecular promedio de entre 5,000 y 20,000 daltons.

28.- El compuesto de la reivindicación 26, caracterizado porque el polietilenglicol se selecciona del grupo que consiste de monometoxipolietilenglicol (MePEG-OH), monometoxipolietilenglicol-succinato (MePEG-S), monometoxipolietilenglicol-succinato de succinimidilo (MePEG-S-NHS), monometoxipolietilenglicol-amina (MePEG-NH₂), monometoxipolietilenglicol-tresilato (MePEG-TRES) y monometoxipolietilenglicol-imidazolil-carbonilo (MePEG-IM).

29.- Un compuesto que se une al receptor de trombopoietina, dicho compuesto tiene:

- (i) un peso molecular menor a 8,000 daltons; y
- (ii) una afinidad de unión al receptor de trombopoietina como lo expresa un IC₅₀ de no más de 100 μ M, en donde dicho compuesto comprende la siguiente secuencia de aminoácidos: (H-IEGPTLRQ(2-Nal)LAARX₁₀)₂ K-NH₂ en donde X₁₀ es sarcosina o β -alanina, y en donde 2-nal es β -(2-naftil)-alanina.

I, David William End, Ph.D. declare and state that :

2. I have a B.S. in biology from Bucknell University, Lewisburg, Pennsylvania, and a Ph.D. in pharmacology from Medical Center College of Virginia, Richmond, Virginia. I completed my postdoctorate in cell biology and neurobiology at the National Institutes of Health, Bethesda, Maryland.

3. I have been working in the field of pharmacology for over twenty six years.

IEGPTLRQ (2-Nal) LAAR-(Sar)
K(NH₂)
IEGPTLRQ (2-Nal) LAAR-(Sar)

5. I understand that the USPTO has asserted that the peptide compound identified above would have been obvious in view of the disclosure of DeSerres et al., Stem Cell 17: 316-326, 1999, which identifies a dimerized peptide compound, referred to as AF15705, which is similar in structure to the 2-Nal-2-dimer, but for the presence of a 1-Nal substituent in place of the 2-Nal substituent. The DeSerres et al. article also discloses a second compound, referred to as GW395058, in which the amino terminus of each monomer of AF15705 is coupled to a polyethylene glycol polymer having a molecular weight of 20,000. For purposes of the following discussion, the unPEGylated dimer will be referred to as "the 1-Nal dimer" and the PEGylated dimer will be referred to as "the PEG-1-Nal dimer".

7. A summary of the results of these comparisons is as follows:

B. Figure 2 shows a comparison of the activity of multiple batches of the two PEGylated dimers, in the same assay as conducted with the two unPEGylated dimers described in A above. Although both PEGylated dimers showed the ability to stimulate the human TPO receptor, all batches of the PEG-2-Nal dimer were substantially less potent than the PEG-1-Nal dimer and had less intrinsic activity (i.e., produced a lower maximal effect).

D. Figures 4 and 5 show dose response curves for the PEG-1-Nal dimer and the PEG-2-Nal dimer in normal mice. Figure 4 shows increases in platelet levels and Figure 5 shows increases in Mean Platelet Volume 6 days following administration. The two compounds each showed the ability to stimulate platelet production, and performed similarly, the latter a somewhat anomalous result in view of the data mentioned in A-C above. However, the data on which these graphs are based show a substantial degree of variability in response, with fairly large standard errors, indicating the possibility that the observed results may not be wholly indicative of the typical response in mice. The anomaly may have been due to a less-than-optimum choice of timing for measuring the response and/or an

9

irregularity in measurement due to an uncorrected tendency for mouse platelets to clump. In fact, other reported studies conducted on the PEG-1-Nal dimer alone in mice show that a four-fold increase in platelet levels is observed with a single subcutaneous dose of 10µg/kg (see, e.g., Boytos et al., *Blood* 1998: 92: 377a attached hereto as Appendix E), whereas Figure 4 herein only shows a four-fold increase in platelets after a single intravenous dose of 100 µg/kg.

8. The results summarized above show that the PEG-2-Nal dimer has a much lower intrinsic activity and potency than the PEG-1-Nal dimer, a result that is surprising and unobvious in itself because of the similarity in structure between the two compounds. This lower intrinsic activity and potency has a potential advantage in the possible reduction or avoidance of side effects that may occur with a highly potent compound. In particular, our clinical development path will require avoidance of platelet excursions to high levels. In addition, however, an added benefit to the lower potency is the relative ease of generating pharmacokinetic (PK) data, a prerequisite for FDA approval, and in the determination of the actual dosing of the product for administration to human subjects. As the discussion below shows, the PEG-2-Nal dimer, with its lower potency, is a far better candidate for clinical use than is the PEG-1-Nal dimer.

9. The PEG-2-Nal dimer was tested in a double-blind, randomized, placebo-controlled study to determine the compound's PK and pharmacodynamic (PD) profiles. The protocol for the study is attached hereto as Appendix F. In brief, separate dosage groups were formed for dosages ranging from 0.375 µg/kg to 3 µg/kg. Each dose group received a single intravenous dose on Day 1, and remained at the clinic until Day 4 for safety monitoring and PK and PD sampling. The starting dose was 15 µg/kg, and successive cohorts planned were 3, 6.25, 12.5, 25 and 50 µg/kg; however, actual dosing schedule was 1.5, 0.75, 3.75, 3.0, and 2.25. The dosage levels were reduced from those originally planned because of the unexpected effect of the PEG-2-Nal dimer on platelet levels, indicating a high level of sensitivity to the compound in humans. Venous blood samples (4.5 mL) were collected predose (within 30 minutes prior to dosing) and at 5 and 30 minutes, and 1, 2, 4, 8 and 12 hours post dose on Day 1, and on Days 2 (24 hours), 3 (48 hours), 4 (72 hours), 6 (120 hours) 9 (192 hours), 12 (264 hours), and 15 days (336 hours). Among other PK parameters evaluated, the mean plasma concentration of the PEG-2-Nal dimer was calculated for all dosage regimens (additional parameters are detailed in Appendix F). Figures 6 and 7, attached hereto, are graphic representations of the mean plasma concentration for each dosage plotted against time on linear and log-linear scales.

10. A review of Figures 6 and 7 shows an interesting and unexpected result: although PK analysis shows that the PEG-2-Nal dimer exhibits dose related kinetics, at doses of 0.75 µg/kg or lower, plasma concentrations of the compound were generally below the limit of quantification of 6.25 ng/mL. Four subjects in the 0.375 µg/kg dose group, and one subject in the 3.0 µg/kg dose group had no quantifiable plasma levels. Plasma concentrations of the PEG-2-Nal dimer were only detectable in the highest dosage regimens (2.25 and 3 µg/kg) over the entire time range of measurement. With lower doses, no PEG-2-Nal dimer was detectable in plasma beyond the 24 hour point, rendering a number of important PK parameters unquantifiable at lower doses.

11. In view of the PK data reported for the PEG-1-Nal dimer in DeSerres et al., this result is particularly unexpected. First, the studies in the DeSerres et al. reference were conducted on animals (rats and monkeys), and the dosage ranges (IV doses of 500-2000 µg/kg for rats, and 10µg/kg for monkeys) were much higher than those that can be used in human studies. In the single dose IV studies of the PEG-1-Nal dimer of DeSerres et al., PK parameters were readily measurable over a prolonged time frame. It was therefore unexpected, based on DeSerres et al., that the amount of the PEG-2-Nal dimer required to achieve normal platelet levels in humans would be achieved at such low dosage levels. More significantly, it would not have been expected that the lower dosages tested would not be detectable in plasma after a very short time period. It is worthy of note that, although DeSerres et al., on page 319, report a lower detection limit in their assay (i.e., 1.6 ng/ml) than was detectable in Applicants' studies (i.e., 6 ng/ml), they also expressly acknowledge that their assay is not specific for measurement of the PEG-1-Nal dimer, and may potentially include metabolic fragments of the PEG-1-Nal dimer. In contrast, Applicants' assay, which uses antibodies directed against the peptide and PEG moieties, is designed to be highly specific for the intact PEGylated dimer. It is critical that bioanalytical assays employed in drug development measure parent compound only.

12. The PK observations on the PEG-2-Nal dimer suggest some surprising benefits for this compound over the PEG-1-Nal dimer in terms of clinical utility:

(a) In order for a clinical candidate to receive FDA approval for use in human subjects, PK data (in particular exposure and elimination) must be provided. (21 CFR 320.21B, copy attached as Appendix G). At a minimum, for confirmation of a compound's safety, it is routinely necessary to demonstrate, at the proposed dosage levels for the compound, (1) that the compound is eliminated from the body; (2) that there is no accumulation of the compound in the body; and (3) how the compound behaves in individuals with renal impairment. This is particularly important with PEGylated compounds, which are generally known to be slowly eliminated from the body. The standard methodology by which elimination is demonstrated is by submission of PK data over a prolonged time period, i.e., for at least the amount of time required to determine the compound's half-life in the body. Therefore, the inability to monitor the presence of the compound in plasma over an extended period after administration virtually precludes the presentation of the necessary safety data. It is also critical to know the safe maximal exposure in order to execute definitive QT cardiovascular safety studies, which the FDA is requiring for this compound.

(b) As shown above, PK data are determinable for the PEG-2-Nal dimer only at the highest tolerated and safe doses administered. Given that the PEG-1-Nal dimer is at least 10 times more potent than the PEG-2-Nal dimer, it is expected that the appropriate dosage range for administration of the PEG-1-Nal dimer will be on the order of 10-fold less than the useful dosage range for the PEG-2-Nal dimer. Based on the present observations of PK data for the PEG-2-Nal dimer, it

is therefore expected that the dosage range of even the highest usable dosage of the PEG-1-Nal will be well below the detectable limit using the available testing methodology. Thus, provision of bioavailability data for the PEG-1-Nal dimer will be at best extremely difficult, if not impossible, making it an undesirable candidate for use in humans.

13. Thus, the observed PK and PD data suggest that, notwithstanding the lower potency of the PEG-2-Nal dimer relative to the PEG-1-Nal dimer, the PEG-2-Nal dimer exhibits a PD profile that confirms its clinical utility, while exhibiting a measurable PK profile that makes it a more desirable candidate for clinical application than the PEG-1-Nal dimer. That the lower potency the PEG-2-Nal dimer should be a better clinical candidate than the highly potent PEG-1-Nal dimer is indeed surprising, and would not have been predicted from any disclosure in the DeSerres et al. reference.

14. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine, or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 5/16/2008

By: David William End

Figures

1-5 from present application

6-7 from 11/400.216

Appendices

Appendix A: Curriculum Vitae

Appendix B: Protocol for biological activity assay

Appendix C: Protocol for rat studies

Appendix D: Protocol for mouse studies

Appendix E: Boytos et al. (1998)

Appendix F: Protocol for PK studies

Appendix G: Copy of 21 CFR 320.21B

FIG. 1

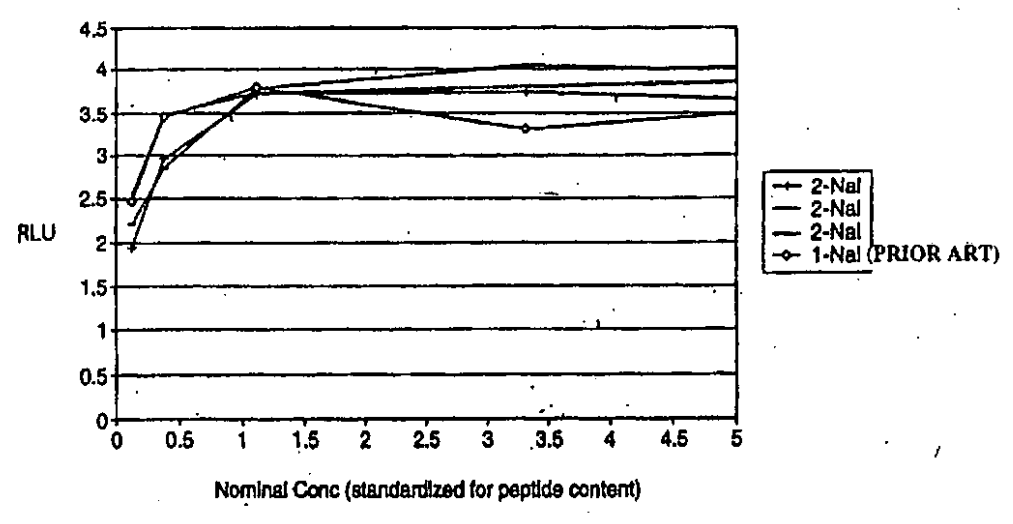


FIG. 2

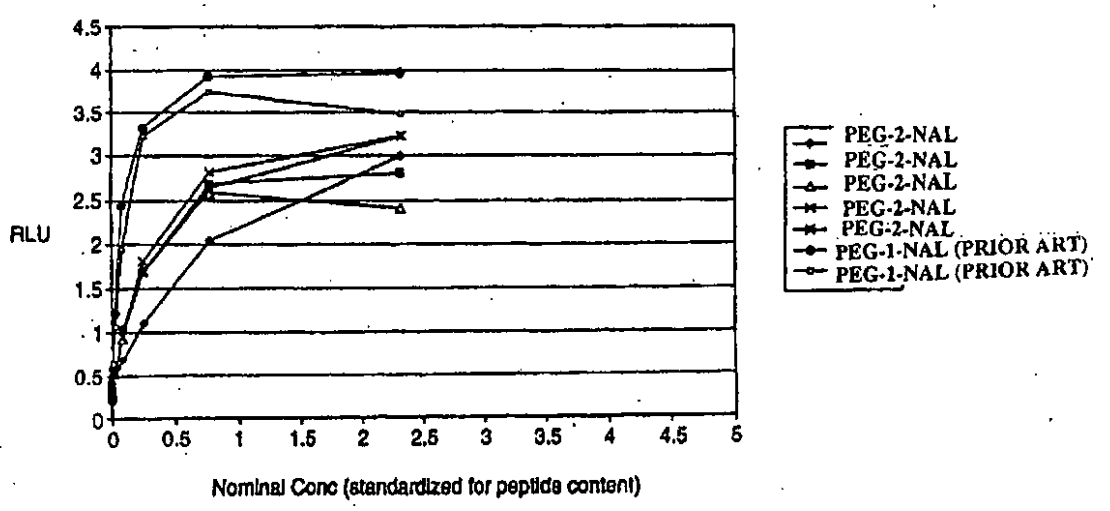


FIG. 3

Summary of Platelet

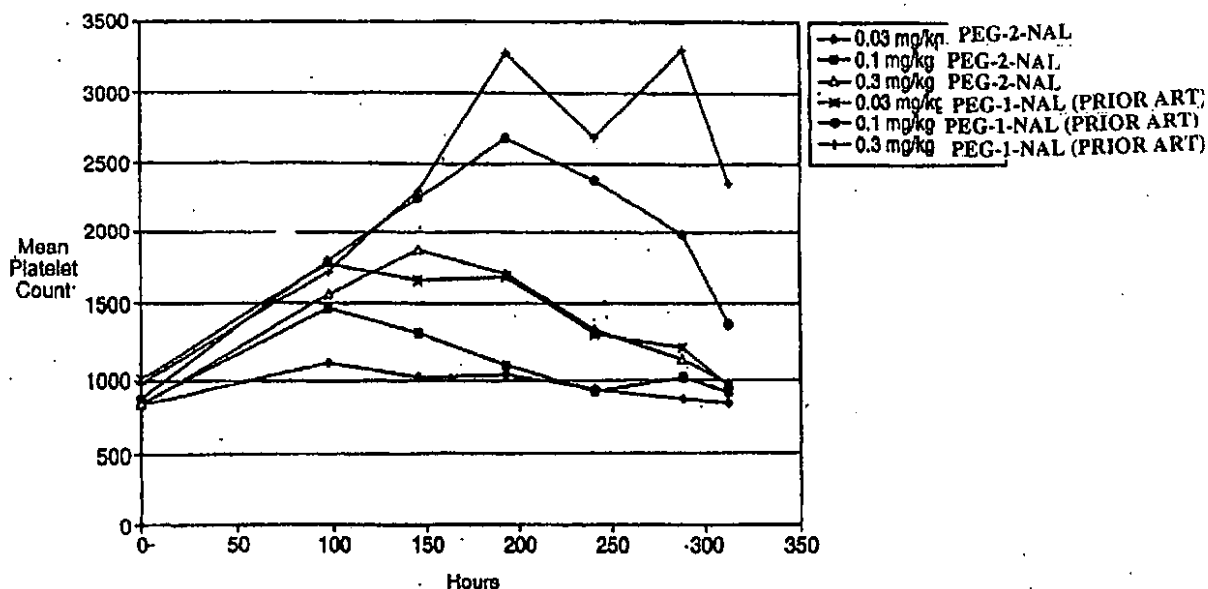


FIG. 4

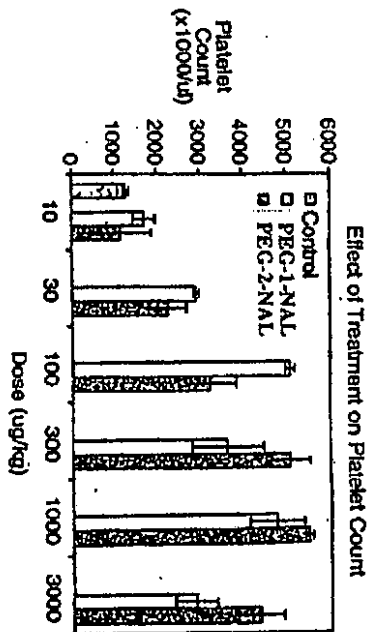
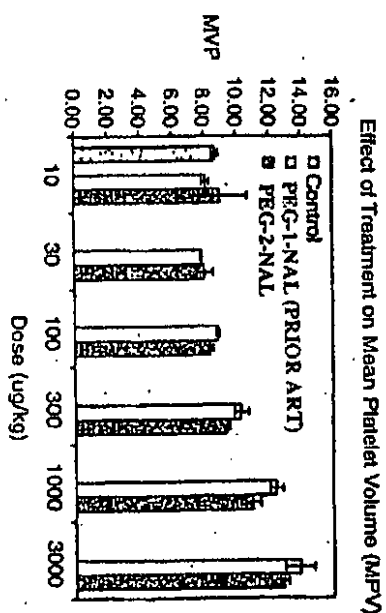
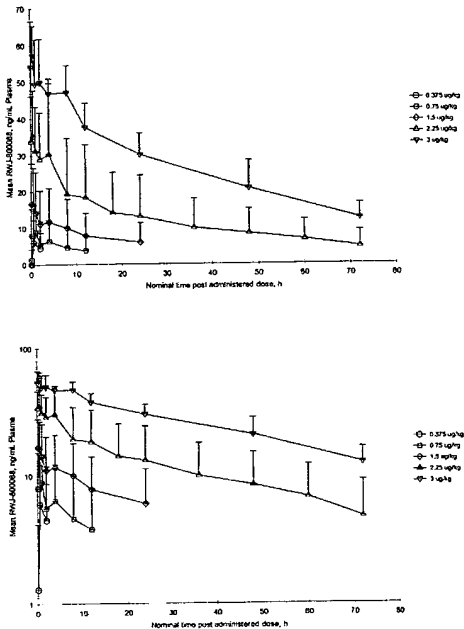


FIG. 5



Figs. 6 & 7: Mean (+SD) PEG-2-Nal plasma concentration versus time plots (linear and log-linear scales)



APPENDIX A

CURRICULUM VITAE

David William End, Ph.D.

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Johnson and Johnson
Pharmaceutical Research and
Development, L.L.C.
Welsh and McKean Roads
Spring House PA, 19477
Phone: 215-628-5852
dend1@prdus.jnj.com

EDUCATION:

Institution	Specialty	Degree	Years
National Institutes of Health Bethesda, MD	Cell Biology Neurobiology	Postdoctorate	1979-1982
Medical College of Virginia Richmond, VA	Pharmacology	Ph.D.	1975-1979
Bucknell University Lewisburg, PA	Biology	B.S., Magna Cum Laude	1971-1975

EXPERIENCE:

Employer	Title	Years
Johnson and Johnson Pharmaceutical Research and Development	Senior Director	2002-present

Responsibilities: Project Leader role to develop project plans from discovery to clinical proof of concept.
Lead highly matrixed intra- and intercompany teams through proof of concept studies and transition to Phase 2B.
Therapeutic areas of oncology supportive care, hematology, pain and urology

Janssen Research Foundation	Senior Research Fellow	1998-2002
	Research Fellow	1993-1998
	Principal Scientist	1990-1993

Responsibilities: Create drug discovery programs in oncology.

Establish and maintain high throughput biochemical screens, secondary cellular tests and animal tumor models. Provide preclinical support for clinical development including biomarker studies. Supervise additional oncology target efforts		
• First team to bring a farnesyl protein transferase inhibitor into clinical development. • Retinoic acid metabolism and a variety of other oncology targets probed.		
<u>Robert Wood Johnson Pharmaceutical Research Institute</u>	Principal Scientist	1989-1990
Responsibilities: Establish drug discovery research in atherosclerosis and antioxidants		
<u>Janssen Research Foundation</u>	Principal Scientist	1987-1989
Responsibilities: Maintain discovery research program in the areas of oncology and inflammation.		
• Retinoic acid metabolism in vivo and in vitro. • Skin inflammation and tumor promotion		
<u>McNeil Pharmaceutical</u>	Principal Scientist Senior Scientist	1986-1987 1982-1986
Responsibilities: Establish drug discovery research projects in the therapeutic areas of oncology and inflammation including input on drug design, primary and secondary screening in the following areas		
• Agents active in dermal inflammation • Inhibitors of prostaglandin and leukotriene biosynthesis • Inhibitors of tyrosine specific protein kinases		
Team Chairman in two project development teams in the area of analgesics. Team representative for development of a cancer chemopreventive retinoid (Fenretinide). Evaluation of outside technologies and business opportunities in the areas of cancer and arthritis. Establishment of tissue culture facilities. Chairman, Radiation Safety Committee.		
<u>National Institute of Child Health and Human Development NIH, Bethesda, MD</u>	Staff Fellow	1981-1982
	Public Health Service Postdoctoral Fellow	1979-1981

Research: Interaction of nerve growth factor (NGF) with epidermal growth factor (EGF), and its role in neuronal development. Methods for the characterization of EGF and NGF receptors. Identification of protein kinase systems coupled to the actions of NGF. Biophysical characterization of the EGF receptor/tyrosine specific protein kinase.		
<u>Medical College of Virginia Richmond, VA</u>	Research Assistant in Primate Colony Predoctoral Fellow	1975-1979 1975-1979
Research: Neurochemical toxicity of chlordecone. Effects of drugs and toxic chemicals on synaptosomal and mitochondrial function		
HONORS AND AWARDS:		
Johnson and Johnson Philip B. Hoffman Award for Outstanding Achievement in Research Janssen Pyramid Award Janssen Pharmaceutica President's Award Public Health Service Postdoctoral Fellowship Virginia Academy of Sciences Research Award National Science Foundation Predoctoral Fellowship A.D. Williams Predoctoral Fellowship Phi Beta Kappa B.S. Magna Cum Laude		1997 1996 1991 1979 1979 1976 1975 1975 1975
INVITED SPEAKER:		
Summit on Molecular Targeted Therapy, Philadelphia, PA. 2003.		
German-Austrian Hematology and Oncology Meeting, Linz, Austria, 2002.		
British Association for Cancer Research Meeting 2001, University of Leeds, U.K.: "Update on farnesyl protein transferase inhibitors", 2001		
German-Austrian Hematology and Oncology Meeting, Graz, Austria, 2000.		
European Organization for Research and Treatment of Cancer Early Drug Development Meeting, Vienna, Austria, 1999. "Farnesyl protein transferase inhibitors-Preclinical aspects."		
American Association for Pharmaceutical Sciences Spring Meeting, 1998: "Farnesyl protein transferase inhibitor (FTI) R115777: from imidazole pharmacophore to oncology clinical trials. "		
Mid-Atlantic Pharmacology Society Symposium on Protein Kinases 1985: "Protein Kinase C as a target for drug discovery".		
PATENTS:		
D. W. End Farnesyl protein transferase inhibitor combinations with antiestrogen agents. EP1365763.		

D. W. End Dosing regimen comprising a farnesyl protein transferase inhibitor for the treatment of cancer. EP1267848

D. W. End Farnesyl protein transferase inhibitors for the treatment of cachexia. WO2002064142..

D. W. End Farnesyl protein transferase inhibitors for the treatment of inflammatory bowel disease. EP 1339407.

D. W. End, M. G. Venet, P. R. Angibaud, and G. C. Sanz Farnesyl transferase 1,2-annelated quinolone enantiomers US20030114471 A1

D. W. End, M. Cools, L. L. Van Wauwe Farnesyl protein transferase inhibitors for treating arthropathies. WO 99-EP454519990630.

R. F. Van Ginckel, J. Floren, D. W. End, and W. B. L. Wouters Farnesyl protein transferase inhibitors with in vivo radiosensitizing properties and use in treating cancer. WO 99-EP454519990603.

D. W. End and M. J. Zelesko (Imidazol-5-yl)methyl-2-quinolone derivatives as inhibitors of smooth muscle cell proliferation. WO 98555124.

D. W. End, M. G. Venet, P. R. Angibaud, and G. C. Sanz Farnesyl transferase inhibiting 2-quinolone derivatives WO 9716443.

PUBLICATIONS:

Invited Reviews:

D.W. End, L. Mevellec, P. Angibaud Farnesyl protein transferase inhibitors: Medicinal Chemistry, molecular mechanisms and progress in the clinic. "Topics in Medicinal Chemistry: Cancer", Springer Series, 2007.

J.E. Karp, S.H. Kaufman, A.A. Adjei, J.E. Lancet, J. J. Wright, and D. W. End Current status of clinical trials of farnesyltransferase inhibitors. Curr. Opinion. Oncology 13: 470-476, 2001.

D. W. End Farnesyl protein transferase inhibitors and other therapies targeting the Ras signal transduction pathway. Investigational New Drugs 17, 241-258, 1999.

Research Manuscripts:

P. Angibaud, L. Mevellec, C. Meyer, X. Bourdez, P. Lezouret, I. Pilatte, V. Poncellet, B. Roux, S. Merillon, D.W. End, J. Van Dun, W. Wouters, Marc Venet Impact on farnesyltransferase inhibition of 4-chlorophenyl moiety replacement in the Zarnestra® series. Eur. J. Med. Chem. 42: 702-714, 2007.

R.A. Lubet, K. Christove, M. You, R. Yao, V.E. Steele, D.W. End, M.M. Juliana and C.J. Grubbs Effect of the farnesyl protein transferase inhibitor R115777 (Zarnestra) on mammary carcinogenesis: prevention, therapy and role of HaRas mutations. Mol. Cancer Ther. 5: 1073-1078, 2006.

W.T. Gunning, P.M. Kramer, R.A. Lubet, V.E. Steele, D.W. End, M.A. Pereira Chemoprevention of benz(a)pyrene-induced lung tumors in mice by the farnesyltransferase inhibitor R115777. Clin. Cancer Res. 9: 1927-1930, 2003.

P. Angibaud, X. Bourdez, A. Devine, D. End, E. Freyne, Y. Ligny, P. Muller, G. Mannens, I. Pilatte, S. Skrzat, G. Smets, J. Van Dub, P. Van Remoorter, M. Venet, W. Wouters 5-imidazolyl-quinolones as farnesyl transferaso inhibitors. Biorg. & Med. Chem. Lett. 13: 1543-1547, 2003.

C. Delmas, C. Heliez, D. End, J. Bonnet, N. Daly-Schveitzer, G. Favre, C. Toulas The farnesyltranferase inhibitor R115777 reverses the resistance of human glioma cell lines to ionizing radiation. International J. Cancer 100: 43-48, 2002.

L. R. Kelland, V. Smith, M. Valenti, L. Patterson, P. A. Clarke, S. Detre, D. End, A. J. Howes, M. Dowsett, P. Workman, and S. R. Johnson Preclinical antitumor activity and pharmacodynamic studies with the farnesyl protein transferase inhibitor R115777 in human breast cancer. Clinical Cancer Res. 7: 3544-3540, 2001.

J. E. Karp, J. E. Lancet, S. H. Kaufman, D. W. End, J. J. Wright, K. Bol, I. Horak, M. L. Tidwell, J. Liesveld, T. J. Kotke, D. Ange, L. Buddharaju, I. Gojo, W. E. Higg, R. T. Belly, R. J. Hohl, M. E. Rybak, A. Thibault, and J. Rosenblatt Clinical and biologic activity of the farnesyltransferase inhibitor R115777 in adults with refractory and relapsed leukemia: A phase I clinical-laboratory correlative trial. Blood 97: 3361-3369, 2001.

David W. End, Gerda Smets, Alison V. Todd, Tanya L. Applegate, Caroline J. Fuery, Patrick Angibaud, Marc Venet, Gerard Sanz, Herve Poignet, Stacy Skrzat, Ann Devine, Walter Wouters, and Charlie Bowden. Characterization of the antitumor effects of the selective farnesyl protein transferase Inhibitor R115777 *In Vivo* and *In Vitro*. Cancer Res. 61: 131-137, 2001.

J. Zujewski, I. Horak, K. Bol, R. Woestenborghs, C. Bowden, D. End, V. Piotovsky, J. Chiao, R. Belly, A. Todd, W. Kopp, D. Kohler, C. Chow, M. Noone, Hakim, G. Larkin, R. Gress, R. Nussenblatt, A. Kremer, K. Cowan Phase I pharmacokinetic study of farnesyl protein transferase inhibitor R115777 in advanced cancer. J. Clin. Oncol. 18, 927-941, 2000.

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D.W. End and T.A. Garrabrant. Characterization of a high affinity interleukin-1 (IL-1) specific binding site in a human synovial sarcoma (Hs431) cell line. Lymphokine Res. 9, 167 - 176, 1990.

C.Y. Ho, W.E. Hageman, R.J. Mohrbacher, and D.W. End. Inhibition of leukotriene and thromboxane biosynthesis by a 9-(4-chlorophenyl) analogue of arachidonic acid. J. Pharm. Sci. 77, 149, 1988.

D.W. End, R.A. Look, T. Garrabrant, and F.J. Persico. Identification of some Interleukin-1 (IL-1) sensitive tissues in vivo using the induction of ornithine decarboxylase following injection of recombinant IL-1β in mice. Lymphokine Res. 6, 4, 1987.

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G. Guroff, G. Dickens, D. End and C. Londres. The action of adenosine analogs on PC12 cells. *J. Neurochem.* 37, 1431-1439, 1981.

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S. G. Skrzat, L. Fanelli, K. Chevalier, P. Jacqmin, L. van Beijsterveldt, R. De Vries, W. Wouters. D.W. End Unexpected Pharmacodynamics of the Farnesyl Protein Transferase Inhibitor (FTI) R115777. *Proc. Am. Assoc. Cancer Res.* 41, 1404, 2000.

S. G. Skrzat, C. R. Bowden and D. W. End. Interaction of farnesyl protein transferase inhibitor (FTI) R115777 with cytotoxic chemotherapeutics in vitro and in vivo. *Proc. Am. Assoc. Cancer Res.* 40: 522, 1999.

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D. W. End, W. Wouters, R. Van Ginckel, R. De Coster, C. Bowden, and T. A. Garabrant. Antitumoral effects of Icarazole in T43 mammary carcinoma cells: Comparison to retinoic acid. *Proc. American Association for Cancer Research.* 32, 1992.

D. W. End, R.A. Look and T. A. Garabrant. lack of effects of levamisole on the antiproliferative effects of 5-fluorouracil in human and murine tumor cells in tissue culture. *Proc. American Assoc. for Cancer Research.* 31, 1991.

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T.A. Garabrant and D.W. End. Localized skin inflammation induced by intradermal injection of human recombinant interleukin-1 (rIL-1). Fourth International Conference of the Inflammation Research Association, Abs. 39, 1988.

D.W. End, J. Uhl, R. Look, T. Garabrant, F. Persico, and E. Mochan. Specificity of rapid phosphorylation-induced by interleukin-1 (IL-1) in human synovial cells. Abstracts American Rheumatism Association 51st Meeting, S82, 1987.

E. Mochan, J. Uhl, R. Look, T. Garabrant, and D. End. Specific changes in new protein synthesis induced by interleukin-1 (IL-1) in human synovial cells. Abstracts American Rheumatism Association 51st Meeting, S82, 1987.

D.W. End, R.A. Look, T. Garabrant, and F.J. Persico. Identification of interleukin-1 (IL-1) sensitive tissues in mice *in vivo* with recombinant IL-1 β . UCLA Symposium, Pharmacology of proteins, Lake Tahoe, CA., 1987.

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D.W. End, N.L. Shaffer, F.J. Persico, and R.A. Look. Examination of the acute inflammation produced by the phorbol ester tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) in mouse skin: Sensitivity to steroidal and nonsteroidal antiinflammatory drugs. Winter Prostaglandin Conference, Keystone, CO., 1984.

N.L. Shaffer, E.A. Balles, F.J. Persico, and D.W. End. Inhibition of tyrosine specific and cAMP-dependent protein kinase systems by quercetin and related bioflavonoids. *Pharmacologist*, 25, 189, 1983.

D.W. End, N.L. Shaffer, E.A. Balles, and F.J. Persico. A rapid in vitro assay for assessing drug effects on the lipoygenase pathway of arachidonic acid metabolism. *Pharmacologist*, 25, 237, 1983.

D.W. End, L. Pevzner, A. Loyd, and G. Guroff. Identification of nerve growth factor (NGF) receptors in chick neural crest cells. *Fed. Proc.* 40, 147, 1982.

D.W. End, N. Tolson, M.-Y. Yu, and G. Guroff. Interactions of 12-O-tetradecanoylphorbol-13-acetate (TPA) with nerve growth factor and epidermal growth factor in rat pheochromocytoma cells. *Fed. Proc.* 40, 252, 1981.

D.W. End, K. Huff, and G. Guroff. Modulation of epidermal growth factor receptors by nerve growth factor in pheochromocytoma cells in culture. *Soc. Neurosci. Abs.* 6, 330, 1980.

D.W. End, R.A. Carchman, R. Ameen, and W.L. Dewey. Disruption of rat brain synaptosomal membranes by the neurotoxic organochlorine insecticide chlordane. *Fed. Proc.* 38, 845, 1978.

D.W. End, R.A. Carchman, and W.L. Dewey. Assessment of the neurotoxicity of Kepone *in vitro*. *Pharmacologist* 20, 188, 1978.

R.A. Carchman, D.W. End, K. Thoursen, W.L. Dewey, and L.S. Harris. The inhibition of growth and macromolecular synthesis in neural cells in tissue culture by delta-9-tetrahydrocannabinol. *Pharmacologist*, 25, 237, 1976.

CONTINUING EDUCATION:
Special Courses

Institution	Course Description	Date
Janssen Research Foundation	Standards of Leadership	2000
QIBCO-BRL	Molecular Biology Techniques	1992
Gaithersburg, MD		
McNeil Pharmaceutical	Quality Improvement	1986
McNeil Pharmaceutical	Immunology	1985
University of Pennsylvania	Management of Technology in a Changing Environment	1983
Wharton		
McNeil Pharmaceutical	HPLC Methods	1982
McNeil Pharmaceutical	Scanticon Management Training	1982
National Institutes of Health	Radiological Health and Radiation Safety	1980

APPENDIX Ø BIOLOGICAL ACTIVITY ASSAY FOR TPO MIMETICS

This assay utilizes murine cells engineered to stably express the human TPO receptor (rhTPO) c-mpl and a luciferase reporter construct driven by the fos promoter. For these studies, plasma spiked with the test compound were incubated with starved reporter cells. In plasma, this activity is measured as a combination of endogenous TPO which cross reacts with the human receptor on the engineered reporter cells as well as rhTPO or $\frac{1 \text{ ngL}}{2 \times 10^6 \text{ cells}}$ that was dosed to the animal.

1. MATERIALS AND METHODS

1.1 GROWING AND MAINTAINING WEHI-3 MURINE LEUKEMIA CELLS

GROWTH PROPERTIES:
WEHI-3B cells grow primarily in suspension, but many cells will attach. Cells will double in number approximately every 24 hours. Growth media is DMEM supplemented with 10% heat inactivated FBS, 2 mM L-Glutamine and 50 ug/ml gentamicin.

SUBCULTURING:
Cell density should be maintained between 0.2×10^6 and 2.0×10^6 cells/mL at 37°C and 5% CO₂. Adherents may be harvested by scraping. Use trypan blue to count viable cells prior to subculturing.

SUPPLIES AND REAGENTS:

- WEHI-3 Cells—ATCC #TIB-68
- DMEM—BioWhittaker #12-604F
- FBS—Mediatech #35-011-C
- L-Glutamine—BioWhittaker #17-605E
- Gentamicin—BioWhittaker #17-518Z
- 0.4% Trypan Blue—BioWhittaker #17-942E
- Corning T150cm² TC Flask, #430825

1.2 PREPARING CONDITIONED MEDIUM FROM WEHI-3 CELLS

- METHOD:**
- Day 1: Seed WEHI-3 at 0.2×10^6 cells/mL in 25mL complete medium into eight T150 flasks (will yield approximately 200mL conditioned medium). This can be scaled up or down as needed.
 - Day 4: Cells should be at about 1.6×10^6 cells/mL. Transfer supernatant to 50mL conical tubes and spin at 450rcf for 3.5 minutes to pellet any cells. Transfer these supernatants to a Nalgene filter apparatus and filter sterilize. Aliquot and freeze at -80°C. Prepare a few microtube aliquots to be tested later for IL-3 concentration. Split some cells back to carry at 0.2×10^6 cells/mL.
 - Approximately every 4 days, collect supernatant as above, seeding fresh flasks to be carried.
 - Pool all supernatants and measure IL-3 concentration using an IL-3 ELISA kit.

- SUPPLIES AND REAGENTS:
- Corning T150cm² TC Flask, #430825
 - Nalgene CN Filter Unit, 0.2um pore size, #450-0020
 - IL-3 ELISA Kit, R&D Systems #M3000

1.3 GROWING AND MAINTAINING BAF/3 CELL LINES

GROWTH PROPERTIES:
BaF/3 cell lines are IL-3-dependent murine lymphoma, including a control line stably expressing the hTPOr only (no luciferase) and a line of BaF/3 cells HuTPO-r fos/lux expressing both the hTPOr gene and luciferase under the control of the fos promoter. These suspension cells double in number approximately every 12 hours.

SUBCULTURING:
Cell density should be maintained between 0.025 x 10⁶ cells/mL to 2.0 x 10⁶ cells/mL at 37°C and 5% CO₂ in DMEM/F12 50/50 mix supplemented with 10% heat inactivated FBS, 10% WEHI-3B conditioned media, 2 mM L-Glutamine and 50 ug/ml gentamicin. Use trypan blue to count viable cells prior to splitting or plating and use passage 10-12 or less for reporter assay.

- SUPPLIES AND REAGENTS:
- BaF/3 cell lines
 - DMEM/F12—Mediatech #10-090-CV
 - FBS—Mediatech #35-011-CV
 - L-Glutamine—BioWhittaker #17-605E
 - Gentamicin—BioWhittaker #17-518Z
 - 0.4% Trypan Blue—BioWhittaker #17-942E
 - Corning T75cm² TC Flask # 430641

1.4 hTPOr REPORTER ASSAY PROTOCOL

Day 1—Starve logarithmically growing BaF/3 cells overnight (about 18 hours) in starving medium (complete medium containing only 0.1% WEHI-3 conditioned medium).

- Spin down 15mL cells at 450rcf for 3.5 minutes at RT; aspirate supernatant
- Resuspend in 15mL starving medium and spin again; aspirate supernatant
- Resuspend in 15mL starving medium and place into a TC flask
- Incubate overnight at 37°C, 5% CO₂.

Day 2—Perform reporter assay:

- Spin 15mL starved cells at 450rcf 3.5 min; aspirate supernatant.
- Add 15mL assay medium (complete medium without WEHI-3 conditioned medium and without phenol red) and spin again; aspirate supernatant.
- Add 5mL assay medium and count viable cells using trypan blue staining.
- Dilute cells to 1 x 10⁶ cells/mL and aliquot 50uL/well into a 96-well white luminometer plate.
- Add 50uL of 2X compound (in assay media or plasma diluted with assay medium) to cells; mix.
- Incubate at 37°C, 5% CO₂ for 2 hours (covered).

- Add 100uL/well Luc-Screen luciferase substrate, pipetting up and down to mix. Incubate reaction 10min at RT.
- Read on luminometer or scintillation counter that has been set up to read extended glow luminescence.

Starving Medium—Complete BaF/3 Medium containing only 0.1% WEHI-3 C.M.

Assay Medium—Complete BaF/3 Medium containing NO WEHI-3 C.M. and NO phenol red (phenol red interferes with luciferase signal).

Luc-Screen luciferase substrate—thaw buffers 1 and 2; mix equal volumes of each buffer and bring to RT in the dark.

- SUPPLIES AND REAGENTS:
- White luminometer plates—Costar #3789
 - Luc-Screen Reporter Assay System—Applied Biosystems #LS1000

Appendix C. Protocol for Preclinical Studies on thrombopoietic activity of TPO mimetics

Normal male Wistar rats were used for the studies. All procedures involving animals were conducted in a animal facility fully accredited by the American Association for Assessment and Accreditation of Laboratory Animal Care an in accordance with The Guide for the Care and Use of Laboratory Animals (NIH).

Male rats, 10 weeks of age, were treated with single intravenous doses of the test compounds at 30, 100 or 300 µg/kg (40 rats/group). At predose, 96, 144, 192, 240, 288, and 312 hours post dose, approximately 0.5 mL of blood was collected by puncture of the jugular vein of unanesthetized rats (5 rats per time point, EDTA as anticoagulant) and platelet counts were assessed using an automated hematology analysis system. Animals were fasted overnight, with water available, prior to each sample collection.

Appendix D. Protocol for measuring dose-dependent effects of TPO mimetics on platelet counts in BALB/c Mice

- Sample preparation:** A 10mg/ml stock solution was prepared for each compound in sterile saline. To dissolve the test compound without foaming, the preparation was placed on a gyratory shaker for 15 minutes at 200 rpm. The stock was filtered using a GV Millex (.22µm) filter. Dosing solutions were then prepared from this stock using sterile saline. Stock and dosing solutions were prepared fresh on the day of use. Residual stock and dosing were archived at -70°C in the dark.
- Test animals:** 65 BALB/c mice 8-9 weeks old (Charles River) were used for the study.
- Dosing:** The groups of mice (5 mice/group) were treated with vehicle (sterile saline, SS, preservative-free 0.9% sodium chloride inj., USP, Lot # 65-376-DK, Abbott Laboratories, North Chicago Illinois) or increasing amounts of either PEG-1-Nal or PEG-2-Nal by iv(bolus) as delineated in the table below. The dose was administered on a per-weight basis (100 µl/10g body weight)

Treatment Groups			
Gp	N	Test Article	Blood Collection
1	5	Vehicle (SS)	Sac 5 mice on Day 6
2	5	PEG-1-Nal	Sac 5 mice on Day 6
3	5	PEG-1-Nal	Sac 5 mice on Day 6
4	5	PEG-1-Nal	Sac 5 mice on Day 6
5	5	PEG-1-Nal	Sac 5 mice on Day 6
6	5	PEG-1-Nal	Sac 5 mice on Day 6
7	5	PEG-1-Nal	Sac 5 mice on Day 6
8	5	PEG-2-Nal	Sac 5 mice on Day 6
9	5	PEG-2-Nal	Sac 5 mice on Day 6
10	5	PEG-2-Nal	Sac 5 mice on Day 6
11	5	PEG-2-Nal	Sac 5 mice on Day 6
12	5	PEG-2-Nal	Sac 5 mice on Day 6
13	5	PEG-2-Nal	Sac 5 mice on Day 6

Gp = Group; Sac = Sacrifice

A viability check was conducted in the a.m. for each day of the study.

On Day 6, all mice in each test group were weighed and then sacrificed using CO₂-asphyxiation and exsanguinations via cardiac puncture. The blood samples were transferred to separate EDTA (lavender-top) microtainers and a hematological evaluation was conducted (i.e., CBC with absolute platelet counts and MPV).

Appendix D: Protocol for study to evaluate pharmacodynamics and pharmacokinetics of single ascending intravenous doses in healthy men

This study was a double-blind, randomized, placebo-controlled study. Each dose group consisted of a separate set of 8 subjects; 6 receive active treatment and 2 placebo. For each dose group, subjects were admitted to the Clinical Research Unit in the morning of the day before dosing (Day -1). On Day 1 subjects received a single intravenous dose of PEG-2-Nal or matching placebo given as a bolus. Subjects remained in the clinic until Day 4 for safety monitoring and PK and PD sampling. Subjects returned to the unit on Days 6, 9, 12, 15, 17, 22, and 29 for additional evaluations. On these days the subjects remained fasted on the morning of the visit. The starting dose was 1.5 µg/kg and successive cohorts planned were 3, 6.25, 12.5, 25, and 50 µg/kg of PEG-2-Nal; the actual dosing schedule was: 1.5, 0.75, 0.375, 3.0 and 2.25 µg/kg.

For all doses the iv administration rate was 0.5 mL/min. However, the volume and time of administration differed depending on the dose administered. For the 1.5, 2.25 and 3.0 µg/kg doses 2 mL was administered over 4 minutes; for the 0.375 and 0.75 µg/kg doses 0.5 mL over 1 minute and 1 mL over 2 minutes was administered, respectively.

Doses were administered corrected for body weight: the dose unit (µg) each subject received is summarized in Table 1. Body weights of subjects receiving PEG-2-Nal ranged from 61.8 to 99.0 kg.

Table 1: Summary of body weights (only subject receiving PEG-2-Nal) and dose unit ranges for each dose group

Dose µg/kg	Mean Body Weight (min-max) kg	Mean Dose Unit* (min-max) µg
0.375	76.3 (70.5-80.0)	28.6 (26.4-3-.0)
0.75	78.0 (61.8-99.0)	58.5 (46.4-74.3)

1.5	76.9 (68.5-83.5)	115 (103-125)
2.25	79.9 (68.7-95.1)	180 (155-214)
3.0	76.1 (64.7-89.5)	228 (194-269)

* Dose unit/µg = (Body weight/kg) x (Dose/µg/kg)

Pharmacokinetic Assessments

A. Sampling and Bioanalysis

Venous blood samples (4.5 mL) were collected predose (within 30 minutes prior to dosing) and at 5 and 30 minutes and 1, 2, 4, 8 and 12 hours post dose on Day 1 and on Days 2 (24 hours), 3 (48 hours), 4 (72 hours), 6 (120 hours), 9 (192 hours), 12 (264 hours), and 15 days (336 hours) post dose for determination of JNJ-26366821 (RWJ-800088) plasma concentrations. The sampling scheme for the 2.25 µg/kg dose was slightly different from that used fro the other dose groups: predose (within 30 minutes prior to dosing) and at 5 and 30 minutes and 1, 2, 4, 8, 12, 18, 24, 36, 48, 60, 72 and 120 hours post dose (i.e. post start of administration).

Urine samples for determination of PEG-2-Nal concentrations were collected predose (within 1 hour prior to dosing taken from urine collected after subjects had been instructed to void their bladder) and over the following intervals: 0-24, 24-48 and 48-72 hours post dose.

Plasma and urine samples were analyzed for PEG-2-Nal using a validated enzyme linked immunosorbent assay (ELISA) method. The assay limit of quantification was 6.25 ng/mL for plasma and urine.

B. Pharmacokinetic Analysis

Where the data allowed, the following plasma pharmacokinetic parameters were determined for PEG-2-Nal using non-compartmental analysis:

- Maximum plasma concentration (C_{max}): obtained directly from the plasma concentration data

- Time of C_{max} (t_{max}): obtained directly from the plasma concentration data
- Elimination half-life ($t_{1/2}$): calculated as $t_{1/2} = (ln 2)/\lambda_z$ where λ_z is estimated by linear regression of the natural log transformed concentration versus time data, in general using at least three data points (excluding the C_{max} value)
- Area under the concentration versus time curve from the 0 to infinity (AUC_{∞}): calculated as $AUC_{\infty} = AUC_{last} + C_{last}/\lambda_z$ where AUC_{last} is the area under the concentration-time curve from time zero to the time of the last quantifiable concentration, C_{last} is the last observed quantifiable concentration, and λ_z is the terminal elimination rate constant.
- AUC_{0-24} : area under the plasma concentration-time curve from time 0 to 24 hours, calculated by linear trapezoidal summation

Where the data allowed, the following urine pharmacokinetic parameters were determined for PEG-2-Nal:

- Cumulative amount of drug excreted in urine over 72 hours post dose (A_{72}): calculated by the summation of the urine volumes of the collection intervals multiplied by the associated urine drug concentrations

Appendix E 1998 ASH Abstract No. 1554

PEGYLATED THROMBOPOIETIN-MIMETIC PEPTIDES ELEVATE BLOOD PLATELETS AND ABLATE THE CHEMOTHERAPY-INDUCED PLATELET NADIR IN MICE. C. M. Boytos, S. K. Rudolph*, C. Merrell*, B. DePrince*, D. Weigl*, M. deSerres*, and G. Lalonde*. Glaxo Wellcome Inc., Research Triangle Park, NC and Affymax Research Institute, Palo Alto, CA.

Single low doses of diPEGylated thrombopoietin-mimetic peptides (GW350805 or GW395058) stimulated platelet production in normal mice and ablated chemotherapy-induced thrombocytopenia in mice. GW350805 and GW395058 were derived from the linear peptide dimers AF13948 and AF15705, respectively, by covalent linkage with 20000 MW polyethylene glycol. PEGylation increased the $t_{1/2}$ for elimination in mice from minutes for the parent peptides AF13948 or AF15705 to hours for the PEGylated peptides, GW350805 and GW395058. The unPEGylated parent peptides, AF13948 and AF15705, elevated blood platelets only 1.2- and 1.5-fold, respectively, at a dose of 1000 ug/kg. In contrast, four-fold increases in peak platelet levels were achieved 6 to 8 days after a single subcutaneous dose of GW350805 (2.5 ug/kg) or GW395058 (10 ug/kg), and platelet levels returned to normal by day 12. Doses of GW350805 and GW395058 as low as 0.1 or 1 ug/kg, respectively, elevated platelets 2-fold above baseline. Platelet levels in normal mice were elevated in a dose-dependent manner with increasing doses of the PEGylated peptides up to doses of 10 ug/kg (GW350805) and 25 ug/kg (GW395058), beyond which stimulation of platelet production decreased. In a murine model of carboplatin-induced thrombocytopenia, a single dose of either PEGylated peptide ablated the platelet nadir. In conclusion, these results suggest that GW350805 and GW395058 have the potential to prevent chemotherapy-induced thrombocytopenia.

Introduction

- AF13948 and AF15705, human thrombopoietin (TPO) receptor agonists, are composed of 29 and 30-amino acid peptide chains, respectively, that are covalently linked through their C-termini. GW350805 and GW395058 are AF13948 and AF15705, respectively, that have been diPEGylated with 20,000 MW polyethylene glycol (PEG) at the N-termini. They are being evaluated for the treatment of chemotherapy-induced thrombocytopenia.
- DiPEGylation of the parent TPO mimetic peptides, AF13948 and AF15705, caused a marked increase in stimulation of thrombocytosis in the mouse.
- The diPEGylated peptide mimetics, GW350805 and GW395058, elevated platelet levels four-fold in mice in a dose-dependent manner.

- Single-dose regimens achieved equivalent maximal platelet stimulation as multiple-dose regimens.
- A single subcutaneous dose of the diPEGylated peptide mimetics ablated the carboplatin-induced platelet nadir.

APPENDIX F

§ 320.21

21 CFR Ch. I (4-1-07 Edition)

Support B—Procedures for Determining the Bioavailability or Bioequivalence of Drug Products

SOURCE: 32 FR 1648, Jan. 7, 1977, unless otherwise noted.

§ 320.21 Requirements for submission of in vivo bioavailability and bioequivalence data.

(a) Any person submitting a full new drug application to the Food and Drug Administration (FDA) shall include in the application either:

- (1) Evidence measuring the in vivo bioavailability of the drug product that is the subject of the application; or
- (2) Information to permit FDA to waive the submission of evidence measuring in vivo bioavailability.

(b) Any person submitting an abbreviated new drug application to FDA shall include in the application either:

- (1) Evidence demonstrating that the drug product that is the subject of the abbreviated new drug application is bioequivalent to the reference listed drug (defined in § 314.3(b) of this chapter); or
- (2) Information to show that the drug product is bioequivalent to the reference listed drug which would permit FDA to waive the submission of evidence demonstrating in vivo bioequivalence as provided in paragraph (f) of this section.

(c) Any person submitting a supplemental application to FDA shall include in the supplemental application the evidence or information set forth in paragraphs (a) and (b) of this section if the supplemental application proposes any of the following changes:

- (1) A change in the manufacturing site or a change in the manufacturing process, including a change in product formulation or dosage strength, beyond the variations provided for in the approved application.
- (2) A change in the labeling to provide for a new indication for use of the drug product, if clinical studies are required to support the new indication for use.
- (3) A change in the labeling to provide for a new dosage regimen or for an additional dosage regimen for a special patient population, e.g., infants, if clinical studies are required to support the new or additional dosage regimen.

ical studies are required to support the new or additional dosage regimen.

(d) FDA may approve a full new drug application, or a supplemental application proposing any of the changes set forth in paragraph (c) of this section, that does not contain evidence of in vivo bioavailability or information to permit waiver of the requirement for in vivo bioavailability data, if all of the following conditions are met.

(1) The application is otherwise approvable.

(2) The application agrees to submit, within the time specified by FDA, either:

- (i) Evidence measuring the in vivo bioavailability and demonstrating the in vivo bioequivalence of the drug product that is the subject of the application; or
- (ii) Information to permit FDA to waive measurement of in vivo bioavailability.

(e) Evidence measuring the in vivo bioavailability and demonstrating the in vivo bioequivalence of a drug product shall be obtained using one of the approaches for determining bioavailability set forth in § 320.24.

(f) Information to permit FDA to waive the submission of evidence measuring the in vivo bioavailability or demonstrating the in vivo bioequivalence shall meet the criteria set forth in § 320.22.

(g) Any person holding an approved full or abbreviated new drug application shall submit to FDA a supplemental application containing new evidence measuring the in vivo bioavailability or demonstrating the in vivo bioequivalence of the drug product that is the subject of the application if notified by FDA that:

- (1) There are data demonstrating that the dosage regimen in the labeling is based on incorrect assumptions or facts regarding the pharmacokinetics of the drug product and that following this dosage regimen could potentially result in subtherapeutic or toxic levels; or
- (2) There are data measuring significant intra-batch and batch-to-batch variability, e.g., plus or minus 25 percent, in the bioavailability of the drug product.

(b) The requirements of this section regarding the submission of evidence measuring the in vivo bioavailability or demonstrating the in vivo bioequivalence apply only to a full or abbreviated new drug application or a supplemental application for a finished dosage formulation.

(57 FR 1799, Apr. 28, 1992, as amended at 57 FR 7072, Dec. 15, 1992)

§ 320.22. Criteria for waiver of evidence of in vivo bioavailability or bioequivalence.

(a) Any person submitting a full or abbreviated new drug application, or a supplemental application proposing any of the changes set forth in § 320.21(c), may request FDA to waive the requirement for the submission of evidence measuring the in vivo bioavailability or demonstrating the in vivo bioequivalence of the drug product that is the subject of the application. An applicant shall submit a request for waiver with the application. Except as provided in paragraph (f) of this section, FDA shall waive the requirement for the submission of evidence of in vivo bioavailability or bioequivalence if the drug product meets any of the provisions of paragraphs (b), (c), (d), or (e) of this section.

(b) For certain drug products, the in vivo bioavailability or bioequivalence of the drug product may be self-evident. FDA shall waive the requirement for the submission of evidence obtained in vivo measuring the bioavailability or demonstrating the bioequivalence of these drug products. A drug product's in vivo bioavailability or bioequivalence may be considered self-evident based on other data in the application if the product meets one of the following criteria:

(1) The drug product:
(i) Is a parenteral solution intended solely for administration by injection, or an ophthalmic or otic solution; and
(ii) Contains the same active and inactive ingredients in the same concentration as a drug product that is the subject of an approved full new drug application or abbreviated new drug application.

(2) The drug product:

(i) Is administered by inhalation as a gas, e.g., a medicinal or an inhalation anesthetic; and
(ii) Contains an active ingredient in the same dosage form as a drug product that is the subject of an approved full new drug application or abbreviated new drug application.

(3) The drug product:

(i) Is a solution for application to the skin, an oral solution, elixir, syrup, injectate, a solution for aerosolization or nebulization, a nasal solution, or similar other solubilized form; and
(ii) Contains an active drug ingredient in the same concentration and dosage form as a drug product that is the subject of an approved full new drug application or abbreviated new drug application; and

(iii) Contains no inactive ingredient or other change in formulation from the drug product that is the subject of the approved full new drug application or abbreviated new drug application that may significantly affect absorption of the active drug ingredient or active moiety for products that are systemically absorbed, or that may significantly affect systemic or local availability for products intended to act locally.

(c) FDA shall waive the requirement for the submission of evidence measuring the in vivo bioavailability or demonstrating the in vivo bioequivalence of a solid oral dosage form (other than a delayed release or extended release dosage form) of a drug product determined to be effective for at least one indication in a Drug Efficacy Study Implementation notice or which is identical, related, or similar to such a drug product under § 318.6 of this chapter unless FDA has evaluated the drug product under the criteria set forth in § 330.33, included the drug product in the Approved Drug Products with Therapeutic Equivalence Evaluations List, and rated the drug product as having a known or potential bioequivalence problem. A drug product so rated reflects a determination by FDA that an in vivo bioequivalence study is required.

(d) For certain drug products, bioavailability may be measured or bioequivalence may be demonstrated by evidence obtained in vitro in lieu of in

Figures

1-5 from present application

6-7 from 11/400,216

Appendices

Appendix A: Curriculum Vitae

Appendix B: Protocol for biological activity assay

Appendix C: Protocol for rat studies

Appendix D: Protocol for mouse studies

Appendix E: Boytos et al. (1998)

Appendix F: Protocol for PK studies

Appendix G: Copy of 21 CFR 320.21B

FIG. 1

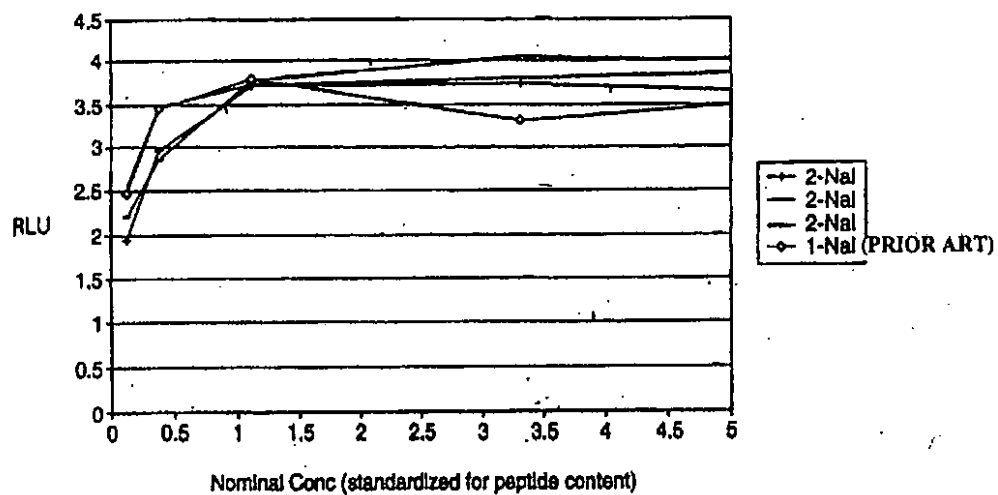


FIG. 2

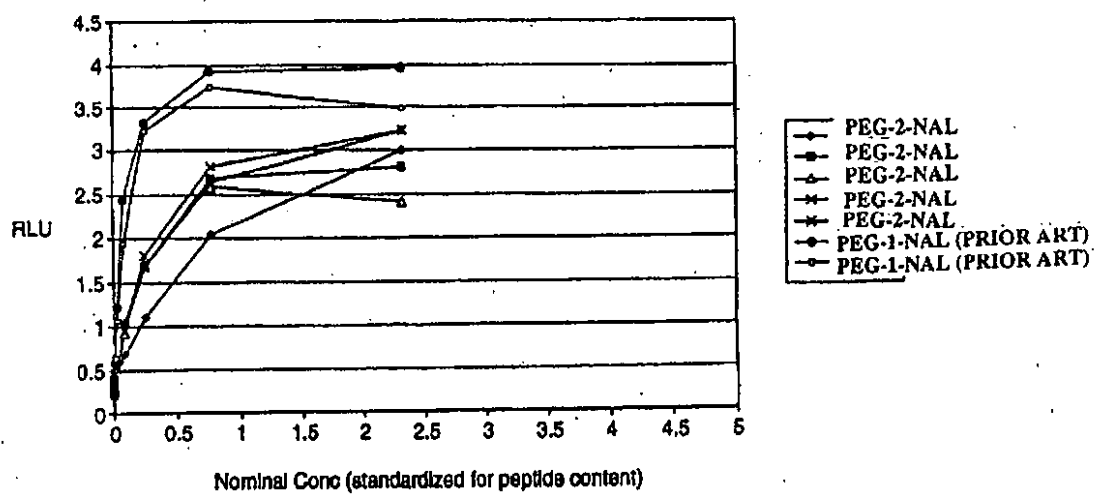


FIG. 3

Summary of Platelet

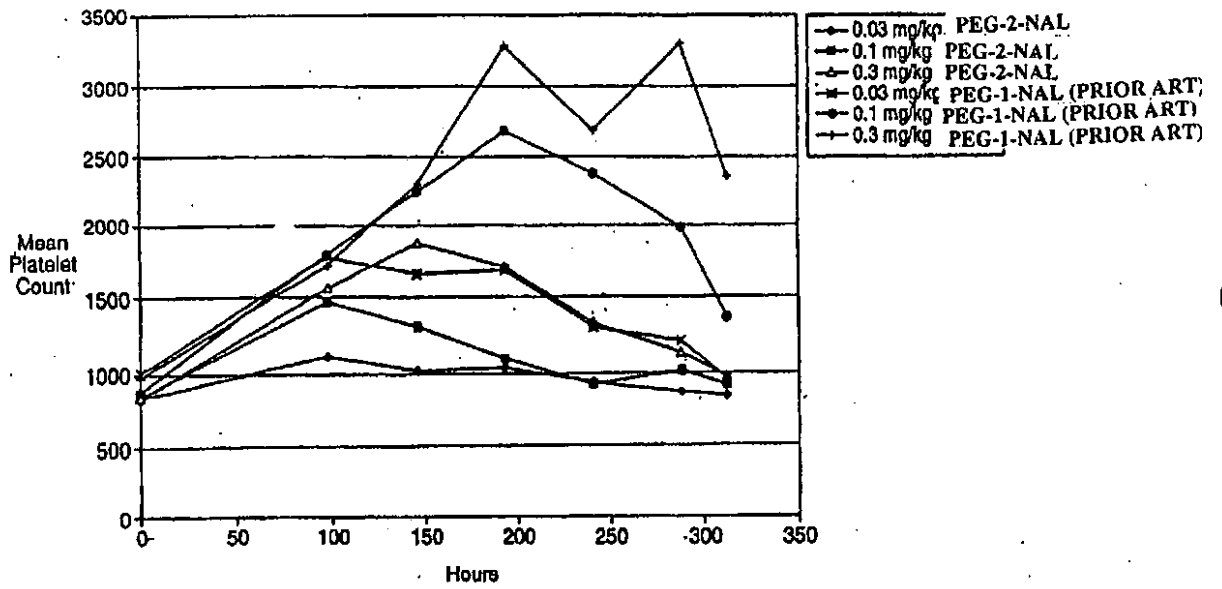


FIG. 4

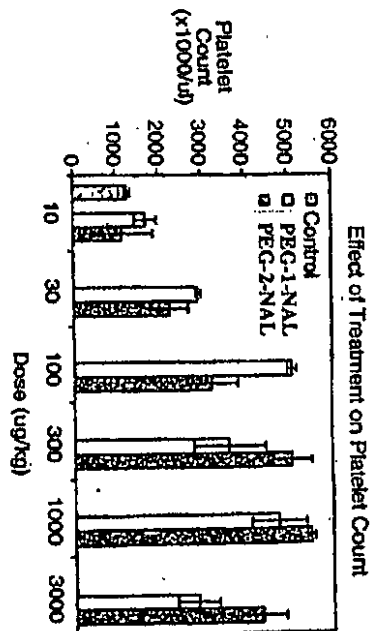
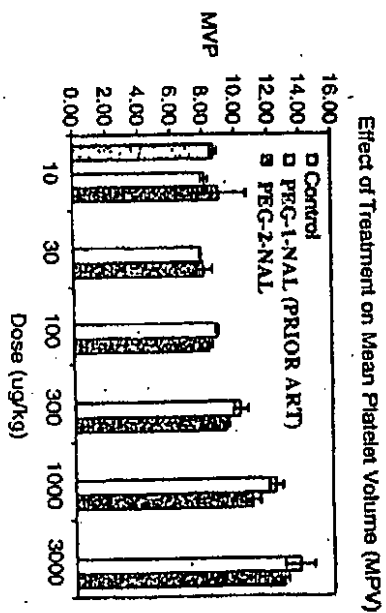
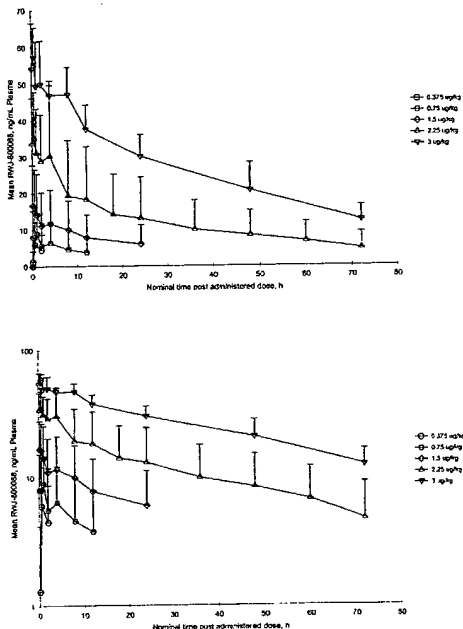


FIG. 5



Figs. 6 & 7: Mean (+SD) PEG-2-Nal plasma concentration versus time plots (linear and log-linear scales)



APPENDIX A

CURRICULUM VITAE

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Johnson and Johnson
Pharmaceutical Research and
Development, L.L.C.
Welsh and McKean Roads
Spring House PA, 19477
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EDUCATION:

Institution	Specialty	Degree	Years
National Institutes of Health Bethesda, MD	Cell Biology Neurobiology	Postdoctorate	1979-1982
Medical College of Virginia Richmond, VA	Pharmacology	Ph.D.	1975-1979
Bucknell University Lewisburg, PA	Biology	B.S., Magna Cum Laude	1971-1975

EXPERIENCE:

Employer	Title	Years
Johnson and Johnson Pharmaceutical Research and Development	Senior Director	2002-present

Responsibilities: Project Leader role to develop project plans from discovery to clinical proof of concept.
Lead highly matrixed intra- and intercompany teams through proof of concept studies and transition to Phase 2B.
Therapeutic areas of oncology supportive care, hematology, pain and urology

Janssen Research Foundation	Senior Research Fellow Research Fellow Principal Scientist	1998-2002 1993-1998 1990-1993
-----------------------------	--	-------------------------------------

Responsibilities: Create drug discovery programs in oncology.

Establish and maintain high throughput biochemical screens, secondary cellular tests and animal tumor models. Provide preclinical support for clinical development including biomarker studies. Supervise additional oncology target efforts		
• First team to bring a farnesyl protein transferase inhibitor into clinical development. • Retinoic acid metabolism and a variety of other oncology targets probed.		
<u>Robert Wood Johnson Pharmaceutical Research Institute</u>	Principal Scientist	1989-1990
Responsibilities:	Establish drug discovery research in atherosclerosis and antioxidants	
<u>Janssen Research Foundation</u>	Principal Scientist	1987-1989
Responsibilities:	Maintain discovery research program in the areas of oncology and inflammation. • Retinoic acid metabolism in vivo and in vitro. • Skin inflammation and tumor promotion	
<u>McNeil Pharmaceutical</u>	Principal Scientist Senior Scientist	1986-1987 1982-1986
Responsibilities:	Establish drug discovery research projects in the therapeutic areas of oncology and inflammation including input on drug design, primary and secondary screening in the following areas • Agents active in dermal inflammation • Inhibitors of prostaglandin and leukotriene biosynthesis • Inhibitors of tyrosine specific protein kinases Team Chairman in two project development teams in the area of analgesics. Team representative for development of a cancer chemopreventive retinoid (Fenretinide). Evaluation of outside technologies and business opportunities in the areas of cancer and arthritis. Establishment of tissue culture facilities. Chairman, Radiation Safety Committee.	
<u>National Institute of Child Health and Human Development NIH, Bethesda, MD</u>	Staff Fellow	1981-1982
	Public Health Service Postdoctoral Fellow	1979-1981

Research:	Interaction of nerve growth factor (NGF) with epidermal growth factor (EGF), and its role in neuronal development. Methods for the characterization of EGF and NGF receptors. Identification of protein kinase systems coupled to the actions of NGF. Biophysical characterization of the EGF receptor/tyrosine specific protein kinase.	
<u>Medical College of Virginia</u> <u>Richmond, VA</u>	Research Assistant in Primate Colony Predoctoral Fellow	1975-1979 1975-1979
Research:	Neurochemical toxicity of chlordane. Effects of drugs and toxic chemicals on synaptosomal and mitochondrial function	
HONORS AND AWARDS:		
Johnson and Johnson Philip B. Hoffman Award for Outstanding Achievement in Research		1997
Janssen Pyramid Award		1996
Janssen Pharmaceutica President's Award		1991
Public Health Service Postdoctoral Fellowship		1979
Virginia Academy of Science Research Award		1979
National Science Foundation Predoctoral Fellowship		1976
A. D. Williams Predoctoral Fellowship		1975
Phi Beta Kappa		1975
B.S. Magna Cum Laude		1975
INVITED SPEAKER:		
Summit on Molecular Targeted Therapy, Philadelphia, PA. 2003.		
German-Austrian Hematology and Oncology Meeting, Lintz, Austria, 2002.		
British Association for Cancer Research Meeting 2001, University of Leeds, U.K.: "Update on farnesyl protein transferase inhibitors", 2001		
German-Austrian Hematology and Oncology Meeting, Graz, Austria, 2000.		
European Organization for Research and Treatment of Cancer Early Drug Development Meeting, Vienna, Austria, 1999: "Farnesyl protein transferase inhibitors-Preclinical aspects."		
American Association for Pharmaceutical Sciences Spring Meeting, 1998: "Farnesyl protein transferase inhibitor (FTI) R115777: from imidazole pharmacophore to oncology clinical trials."		
Mid-Atlantic Pharmacology Society Symposium on Protein Kinases 1985: "Protein Kinase C as a target for drug discovery".		
PATENTS:		
D. W. End Farnesyl protein transferase inhibitor combinations with antiestrogen agents. EP1365763.		

D. W. End Dosing regimen comprising a farnesyl protein transferase inhibitor for the treatment of cancer. EP1267848

D. W. End Farnesyl protein transferase inhibitors for the treatment of cachexia. WO2002064142..

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D. W. End, M. G. Venet, P. R. Angibaud, and G. C. Sanz Farnesyl transferase 1,2-annelated quinolone enantiomers US20030114471 A1

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D. W. End Farnesyl protein transferase inhibitors and other therapies targeting the Ras signal transduction pathway. Investigational Now Drugs 17, 241-258, 1999.

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R.A. Lubet, K. Christove, M. You, R. Yao, V.E. Steele, D.W. End, M.M. Juliana and C.J. Grubbs Effect of the farnesyl protein transferase inhibitor R115777 (Zarnestra) on mammary carcinogenesis: prevention, therapy and role of HaRas mutations. Mol. Cancer Ther. 5: 1073-1078, 2006.

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L. R. Kelland, V. Smith, M. Valenti, L. Patterson, P. A. Clarke, S. Detre, D. End, A. J. Howes, M. Dowsett, P. Workman, and S. R. Johnson Preclinical antitumor activity and pharmacodynamic studies with the farnesyl protein transferase inhibitor R115777 in human breast cancer. Clinical Cancer Res. 7: 35444-35450, 2001.

J. E. Karp, J. E. Lancet, S. H. Kaufman, D. W. End, J. J. Wright, K. Bol, I. Horak, M. L. Tidwell, J. Liesveld, T. J. Kotke, D. Ange, L. Buddharaju, I. Gojo, W. E. Higg, R. T. Bely, R. J. Hohl, M. E. Rybak, A. Thibault, and J. Rosenblatt Clinical and biologic activity of the farnesyltransferase inhibitor R115777 in adults with refractory and relapsed leukemia: A phase I clinical-laboratory correlative trial. Blood 97: 3361-3369, 2001.

David W. End, Gerda Smets, Alison V. Todd, Tanya L. Applegate, Caroline J. Fueny, Patrick Angibaud, Marc Venet, Gerard Sanz, Herve Poignet, Stacy Skrzat, Ann Devine, Walter Wouters, and Charlie Bowden. Characterization of the antitumor effects of the selective farnesyl protein transferase inhibitor R115777 *In Vivo* and *In Vitro*. Cancer Res. 61: 131-137, 2001.

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CONTINUING EDUCATION:
Special Courses

Institution	Course Description	Date
Janssen Research Foundation	Standards of Leadership	2000
GIBCO-BRL	Molecular Biology Techniques	1992
Gaithersburg, MD		
McNeil Pharmaceutical	Quality Improvement	1986
McNeil Pharmaceutical	Immunology	1985
University of Pennsylvania	Management of Technology in a Changing Environment	1983
Wharton		
McNeil Pharmaceutical	HPLC Methods	1982
McNeil Pharmaceutical	Scanticon Management Training	1982
National Institutes of Health	Radiological Health and Radiation Safety	1980

APPENDIX 6 BIOLOGICAL ACTIVITY ASSAY FOR TPO MIMETICS

This assay utilizes murine cells engineered to stably express the human TPO receptor (hTPO) c-mpl and a luciferase reporter construct driven by the fos promoter. For these studies, plasma spiked with the test compound were incubated with starved reporter cells. In plasma, this activity is measured as a combination of endogenous TPO which cross reacts with the human receptor on the engineered reporter cells as well as hTPO or that was dosed to the animal.



1. MATERIALS AND METHODS

1.1 GROWING AND MAINTAINING WEHI-3 MURINE LEUKEMIA CELLS

GROWTH PROPERTIES:
WEHI-3B cells grow primarily in suspension, but many cells will attach. Cells will double in number approximately every 24 hours. Growth media is DMEM supplemented with 10% heat inactivated FBS, 2 mM L-Glutamine and 50 ug/ml gentamicin.

SUBCULTURING:
Cell density should be maintained between 0.2 x 10⁶ and 2.0 x 10⁶ cells/mL at 37°C and 5% CO₂. Adherents may be harvested by scraping. Use trypan blue to count viable cells prior to subculturing.

- SUPPLIES AND REAGENTS:
- WEHI-3 Cells—ATCC #TIB-68
 - DMEM—BioWhittaker #12-604F
 - FBS—Mediatech #35-011-C
 - L-Glutamine—BioWhittaker #17-605E
 - Gentamicin—BioWhittaker #17-518Z
 - 0.4% Trypan Blue—BioWhittaker #17-942E
 - Corning T150cm² TC Flask, #430825

1.2 PREPARING CONDITIONED MEDIUM FROM WEHI-3 CELLS

- METHOD:
- Day 1: Seed WEHI-3 at 0.2 x 10⁶ cells/mL in 25mL complete medium into eight T150 flasks (will yield approximately 200mL conditioned medium). This can be scaled up or down as needed.
 - Day 4: Cells should be at about 1.6 x 10⁶ cells/mL. Transfer supernatant to 50mL conical tubes and spin at 450rcf for 3.5 minutes to pellet any cells. Transfer these supernatants to a Nalgene filter apparatus and filter sterilize. Aliquot and freeze at -80°C. Prepare a few microtube aliquots to be tested later for IL-3 concentration. Split some cells back to carry at 0.2 x 10⁶ cells/mL.
 - Approximately every 4 days, collect supernatant as above, seeding fresh flasks to be carried.
 - Pool all supernatants and measure IL-3 concentration using an IL-3 ELISA kit.

- SUPPLIES AND REAGENTS:
- Corning T150cm² TC Flask, #430825
 - Nalgene CN Filter Unit, 0.2um pore size, #450-0020
 - IL-3 ELISA Kit, R&D Systems #M3000

1.3 GROWING AND MAINTAINING BAF/3 CELL LINES

GROWTH PROPERTIES:
BaF/3 cell lines are IL-3-dependent murine lymphoma, including a control line stably expressing the hTPOr only (no luciferase) and a line of BaF/3 cells HuTPO-r fos/lux expressing both the hTPOr gene and luciferase under the control of the fos promoter. These suspension cells double in number approximately every 12 hours.

SUBCULTURING:
Cell density should be maintained between 0.025 x 10⁶ cells/mL to 2.0 x 10⁶ cells/mL at 37°C and 5% CO₂ in DMEM/F12 50/50 mix supplemented with 10% heat inactivated FBS, 10% WEHI-3B conditioned media, 2 mM L-Glutamine and 50 ug/ml gentamicin. Use trypan blue to count viable cells prior to splitting or plating and use passage 10-12 or less for reporter assay.

- SUPPLIES AND REAGENTS:
- BaF/3 cell lines
 - DMEM/F12—Mediatech #10-090-CV
 - FBS—Mediatech #35-011-CV
 - L-Glutamine—BioWhittaker #17-605E
 - Gentamicin—BioWhittaker #17-518Z
 - 0.4% Trypan Blue—BioWhittaker #17-942E
 - Corning T75cm² TC Flask # 430641

1.4 hTPOr REPORTER ASSAY PROTOCOL

Day 1—Starve logarithmically growing BaF/3 cells overnight (about 18 hours) in starving medium (complete medium containing only 0.1% WEHI-3 conditioned medium).

- Spin down 15mL cells at 450rcf for 3.5 minutes at 4°C; aspirate supernatant
- Resuspend in 15mL starving medium and spin again; aspirate supernatant
- Resuspend in 15mL starving medium and place into a TC flask
- Incubate overnight at 37°C, 5% CO₂.

Day 2—Perform reporter assay:

- Spin 15mL starved cells at 450rcf 3.5 min; aspirate supernatant.
- Add 15mL assay medium (complete medium without WEHI-3 conditioned medium and without phenol red) and spin again; aspirate supernatant.
- Add 5mL assay medium and count viable cells using trypan blue staining.
- Dilute cells to 1 x 10⁶ cells/mL and aliquot 50uL/well into a 96-well white luminometer plate.
- Add 50uL of 2X compound (in assay media or plasma diluted with assay medium) to cells: mix.
- Incubate at 37°C, 5% CO₂ for 2 hours (covered).

- Add 100uL/well Luc-Screen luciferase substrate, pipetting up and down to mix. Incubate reaction 10min at RT.
- Read on luminometer or scintillation counter that has been set up to read extended glow luminescence.

Starving Medium—Complete BaF/3 Medium containing only 0.1% WEHI-3 C.M.

Assay Medium—Complete BaF/3 Medium containing NO WEHI-3 C.M. and NO phenol red (phenol red interferes with luciferase signal).

Luc-Screen luciferase substrate—thaw buffers 1 and 2; mix equal volumes of each buffer and bring to RT in the dark.

- SUPPLIES AND REAGENTS:
- White luminometer plates—Costar #3789
 - Luc-Screen Reporter Assay System—Applied Biosystems #LS1000

Appendix C Protocol for Preclinical Studies on thrombopoietic activity of TPO mimetics

Normal male Wistar rats were used for the studies. All procedures involving animals were conducted in a animal facility fully accredited by the American Association for Assessment and Accreditation of Laboratory Animal Care an in accordance with The Guide for the Care and Use of Laboratory Animals (NIH).

Male rats, 10 weeks of age, were treated with single intravenous doses of the test compounds at 30, 100 or 300 µg/kg (40 rats/group). At predose, 96, 144, 192, 240, 288, and 312 hours post dose, approximately 0.5 mL of blood was collected by puncture of the jugular vein of unanesthetized rats (5 rats per time point, EDTA as anticoagulant) and platelet counts were assessed using an automated hematology analysis system. Animals were fasted overnight, with water available, prior to each sample collection.

Appendix D Protocol for measuring dose-dependent effects of TPO mimetics on platelet counts in BALB/c Mice

1. **Sample preparation:** A 10mg/ml stock solution was prepared for each compound in sterile saline. To dissolve the test compound without foaming, the preparation was placed on a gyrotary shaker for 15 minutes at 200 rpm. The stock was filtered using a GV Millex (.22µm) filter. Dosing solutions were then prepared from this stock using sterile saline. Stock and dosing solutions were prepared fresh on the day of use. Residual stock and dosing were archived at -70°C in the dark.
2. **Test animals:** 65 BALB/c mice 8-9 weeks old (Charles River) were used for the study.
3. **Dosing:** The groups of mice (5 mice/group) were treated with vehicle (sterile saline, SS, preservative-free 0.9% sodium chloride inj., USP, Lot # 65-376-DK, Abbott Laboratories, North Chicago Illinois) or increasing amounts of either PEG-1-Nal or PEG-2-Nal by iv(bolus) as delineated in the table below. The dose was administered on a per-weight basis (100 µl/10g body weight)

Treatment Groups:				
Gp	N	Test Article	Dose (iv, Day 0	Blood Collection
1	5	Vehicle (SS)	100ul/10g BW	Sac 5 mice on Day 6
2	5	PEG-1-Nal	10ug/kg	Sac 5 mice on Day 6
3	5	PEG-1-Nal	30ug/kg	Sac 5 mice on Day 6
4	5	PEG-1-Nal	100ug/kg	Sac 5 mice on Day 6
5	5	PEG-1-Nal	300ug/kg	Sac 5 mice on Day 6
6	5	PEG-1-Nal	1000ug/kg	Sac 5 mice on Day 6
7	5	PEG-1-Nal	3000ug/kg	Sac 5 mice on Day 6
8	5	PEG-2-Nal	10ug/kg	Sac 5 mice on Day 6
9	5	PEG-2-Nal	30ug/kg	Sac 5 mice on Day 6
10	5	PEG-2-Nal	100ug/kg	Sac 5 mice on Day 6
11	5	PEG-2-Nal	300ug/kg	Sac 5 mice on Day 6
12	5	PEG-2-Nal	1000ug/kg	Sac 5 mice on Day 6
13	5	PEG-2-Nal	3000ug/kg	Sac 5 mice on Day 6
Gp = Group; Sac = Sacrifice				

A viability check was conducted in the a.m. for each day of the study.

On Day 6, all mice in each test group were weighed and then sacrificed using CO₂-asphyxiation and exsanguinations via cardiac puncture. The blood samples were transferred to separate EDTA (lavender-top) microtainers and a hematological evaluation was conducted (i.e., CBC with absolute platelet counts and MPV).

Appendix D: Protocol for study to evaluate pharmacodynamics and pharmacokinetics of single ascending intravenous doses in healthy men

This study was a double-blind, randomized, placebo-controlled study. Each dose group consisted of a separate set of 8 subjects; 6 receive active treatment and 2 placebo. For each dose group, subjects were admitted to the Clinical Research Unit in the morning of the day before dosing (Day -1). On Day 1 subjects received a single intravenous dose of PEG-2-Nal or matching placebo given as a bolus. Subjects remained in the clinic until Day 4 for safety monitoring and PK and PD sampling. Subjects returned to the unit on Days 6, 9, 12, 15, 17, 22, and 29 for additional evaluations. On these days the subjects remained fasted on the morning of the visit. The starting dose was 1.5 µg/kg and successive cohorts planned were 3, 6.25, 12.5, 25, and 50 µg/kg of PEG-2-Nal; the actual dosing schedule was: 1.5, 0.75, 0.375, 3.0 and 2.25 µg/kg.

For all doses the iv administration rate was 0.5 mL/min. However, the volume and time of administration differed depending on the dose administered. For the 1.5, 2.25 and 3.0 µg/kg doses 2 mL was administered over 4 minutes; for the 0.375 and 0.75 µg/kg doses 0.5 mL over 1 minute and 1 mL over 2 minutes was administered, respectively.

Doses were administered corrected for body weight: the dose unit (µg) each subject received is summarized in Table 1. Body weights of subjects receiving PEG-2-Nal ranged from 61.8 to 99.0 kg.

Table 1: Summary of body weights (only subject receiving PEG-2-Nal) and dose unit ranges for each dose group

Dose µg/kg	Mean Body Weight (min-max) kg	Mean Dose Unit* (min-max) µg
0.375	76.3 (70.5-80.0)	28.6 (26.4-3.0)
0.75	78.0 (61.8-99.0)	58.5 (46.4-74.3)

1.5	76.9 (68.5-83.5)	115 (103-125)
2.25	79.9 (68.7-95.1)	180 (155-214)
3.0	76.1 (64.7-89.5)	228 (194-269)

* Dose unit/µg = (Body weight/kg) x (Dose/µg/kg)

Pharmacokinetic Assessments

A. Sampling and Bioanalysis

Venous blood samples (4.5 mL) were collected predose (within 30 minutes prior to dosing) and at 5 and 30 minutes and 1, 2, 4, 8 and 12 hours post dose on Day 1 and on Days 2 (24 hours); 3 (48 hours), 4 (72 hours), 6 (120 hours), 9 (192 hours), 12 (264 hours), and 15 days (336 hours) post dose for determination of JNJ-26366821 (RWJ-800088) plasma concentrations. The sampling scheme for the 2.25 µg/kg dose was slightly different from that used for the other dose groups: predose (within 30 minutes prior to dosing) and at 5 and 30 minutes and 1, 2, 4, 8, 12, 18, 24, 36, 48, 60, 72 and 120 hours post dose (i.e. post start of administration).

Urine samples for determination of PEG-2-Nal concentrations were collected predose (within 1 hour prior to dosing taken from urine collected after subjects had been instructed to void their bladder) and over the following intervals: 0-24, 24-48 and 48-72 hours post dose.

Plasma and urine samples were analyzed for PEG-2-Nal using a validated enzyme linked immunosorbent assay (ELISA) method. The assay limit of quantification was 6.25 ng/mL for plasma and urine.

B. Pharmacokinetic Analysis

Where the data allowed, the following plasma pharmacokinetic parameters were determined for PEG-2-Nal using non-compartmental analysis:

- Maximum plasma concentration (C_{max}): obtained directly from the plasma concentration data

- Time of C_{max} (t_{max}): obtained directly from the plasma concentration data
- Elimination half-life ($t_{1/2}$): calculated as $t_{1/2} = (1n\ 2)/\lambda_z$ where λ_z is estimated by linear regression of the natural log transformed concentration versus time data, in general using at least three data points (excluding the C_{max} value)
- Area under the concentration versus time curve from the 0 to infinity (AUC_{∞}): calculated as $AUC_{\infty} = AUC_{last} + C_{last}/\lambda_z$ where AUC_{last} is the area under the concentration-time curve from time zero to the time of the last quantifiable concentration, C_{last} is the last observed quantifiable concentration, and λ_z is the terminal elimination rate constant.
- AUC_{0-24} : area under the plasma concentration-time curve from time 0 to 24 hours, calculated by linear trapezoidal summation

Where the data allowed, the following urine pharmacokinetic parameters were determined for PEG-2-Nal:

- Cumulative amount of drug excreted in urine over 72 hours post dose (A_{e72}): calculated by the summation of the urine volumes of the collection intervals multiplied by the associated urine drug concentrations

Appendix E 1998 ASH Abstract No. 1554

PEGYLATED THROMBOPOIETIN-MIMETIC PEPTIDES ELEVATE BLOOD PLATELETS AND ABLATE THE CHEMOTHERAPY-INDUCED PLATELET NADIR IN MICE C. M. Boytos, S. K. Rudolph*, C. Mettall*, R. DePrince*, D. Weigl*, M. deSerres*, and G. Lalonde*. Glaxo Wellcome Inc., Research Triangle Park, NC and Affymax Research Institute, Palo Alto, CA.

Single low doses of diPEGylated thrombopoietin-mimetic peptides (GW350805 or GW395058) stimulated platelet production in normal mice and ablated chemotherapy-induced thrombocytopenia in mice. GW350805 and GW395058 were derived from the linear peptide dimers AF13948 and AF15705, respectively, by covalent linkage with 20000 MW polyethylene glycol. PEGylation increased the t 1/2 for elimination in mice from minutes for the parent peptides AF13948 or AF15705 to hours for the PEGylated peptides, GW350805 and GW395058. The unPEGylated parent peptides, AF13948 and AF15705, elevated blood platelets only 1.2- and 1.5-fold, respectively, at a dose of 1000 ug/kg. In contrast, four-fold increases in peak platelet levels were achieved 6 to 8 days after a single subcutaneous dose of GW350805 (2.5 ug/kg) or GW395058 (10 ug/kg), and platelet levels returned to normal by day 12. Doses of GW350805 and GW395058 as low as 0.1 or 1 ug/kg, respectively, elevated platelets 2-fold above baseline. Platelet levels in normal mice were elevated in a dose-dependent manner with increasing doses of the PEGylated peptides up to doses of 10 ug/kg (GW350805) and 25 ug/kg (GW395058), beyond which stimulation of platelet production decreased. In a murine model of carboplatin-induced thrombocytopenia, a single dose of either PEGylated peptide ablated the platelet nadir. In conclusion, these results suggest that GW350805 and GW395058 have the potential to prevent chemotherapy-induced thrombocytopenia.

Introduction

- AF13948 and AF15705, human thrombopoietin (TPO) receptor agonists, are composed of 29 and 30-amino acid peptide chains, respectively, that are covalently linked through their C-termini. GW350805 and GW395058 are AF13948 and AF15705, respectively, that have been diPEGylated with 20,000 MW polyethylene glycol (PEG) at the N-termini. They are being evaluated for the treatment of chemotherapy-induced thrombocytopenia.
- DiPEGylation of the parent TPO mimetic peptides, AF13948 and AF15705, caused a marked increase in stimulation of thrombocytosis in the mouse.
- The diPEGylated peptide mimetics, GW350805 and GW395058, elevated platelet levels four-fold in mice in a dose-dependent manner.

- Single-dose regimens achieved equivalent maximal platelet stimulation as multiple-dose regimens.
- A single subcutaneous dose of the diPEGylated peptide mimetics ablated the carboplatin-induced platelet nadir.

APPENDIX F

§ 320.21

21 CFR Ch. I (4-1-07 Edition)

Subpart B—Procedures for Determining the Bioavailability or Bioequivalence of Drug Products

SOURCE: 42 FR 1548, Jan. 7, 1977, unless otherwise noted.

§ 320.21 Requirements for submission of in vivo bioavailability and bioequivalence data.

(a) Any person submitting a full new drug application to the Food and Drug Administration (FDA) shall include in the application either:

- (1) Evidence measuring the in vivo bioavailability of the drug product that is the subject of the application; or
- (2) Information to permit FDA to waive the submission of evidence measuring in vivo bioavailability.

(b) Any person submitting an abbreviated new drug application to FDA shall include in the application either:

- (1) Evidence demonstrating that the drug product that is the subject of the abbreviated new drug application is bioequivalent to the reference listed drug (defined in 101.5(b) of this chapter); or
- (2) Information to show that the drug product is bioequivalent to the reference listed drug which would permit FDA to waive the submission of evidence demonstrating in vivo bioequivalence as provided in paragraph (f) of this section.

(c) Any person submitting a supplemental application to FDA shall include in the supplemental application the evidence or information set forth in paragraphs (a) and (b) of this section if the supplemental application proposes any of the following changes:

- (1) A change in the manufacturing site or a change in the manufacturing process, including a change in product formulation or dosage strength, beyond the variations provided for in the approved application.
- (2) A change in the labeling to provide for a new indication for use of the drug product, if clinical studies are required to support the new indication for use.
- (3) A change in the labeling to provide for a new dosage regimen or for an additional dosage regimen for a special patient population, e.g., infants, if clinical

ical studies are required to support the new or additional dosage regimen.

(d) FDA may approve a full new drug application, or a supplemental application proposing any of the changes set forth in paragraph (c) of this section, that does not contain evidence of in vivo bioavailability or information to permit waiver of the requirement for in vivo bioavailability data, if all of the following conditions are met.

- (1) The application is otherwise approvable.
- (2) The application agrees to submit, within the time specified by FDA, either:

- (i) Evidence measuring the in vivo bioavailability and demonstrating the in vivo bioequivalence of the drug product that is the subject of the application; or
- (ii) Information to permit FDA to waive measurement of in vivo bioavailability.

(e) Evidence measuring the in vivo bioavailability and demonstrating the in vivo bioequivalence of a drug product shall be obtained using one of the approaches for determining bioavailability set forth in § 320.24.

(f) Information to permit FDA to waive the submission of evidence measuring the in vivo bioavailability or demonstrating the in vivo bioequivalence shall meet the criteria set forth in § 320.22.

(g) Any person holding an approved full or abbreviated new drug application shall submit to FDA a supplemental application containing new evidence measuring the in vivo bioavailability or demonstrating the in vivo bioequivalence of the drug product that is the subject of the application if notified by FDA that:

- (1) There are data demonstrating that the dosage regimen in the labeling is based on incorrect assumptions or facts regarding the pharmacokinetics of the drug product and that following this dosage regimen could potentially result in subtherapeutic or toxic levels; or
- (2) There are data measuring significant intra-batch and batch-to-batch variability, e.g., plus or minus 25 percent, in the bioavailability of the drug product.

(b) The requirements of this section regarding the submission of evidence measuring the in vivo bioavailability or demonstrating the in vivo bioequivalence apply only to a full or abbreviated new drug application or a supplemental application for a finished dosage formulation.

(57 FR 1996, Apr. 28, 1992, as amended at 57 FR 10725, Dec. 10, 1992)

§ 320.22 Criteria for waiver of evidence of in vivo bioavailability or bioequivalence.

(a) Any person submitting a full or abbreviated new drug application, or a supplemental application proposing any of the changes set forth in § 320.21(c), may request FDA to waive the requirement for the submission of evidence measuring the in vivo bioavailability of the drug product that is the subject of the application. An applicant shall submit a request for waiver with the application. Except as provided in paragraph (f) of this section, FDA shall waive the requirement for the submission of evidence of in vivo bioavailability or bioequivalence if the drug product meets any of the provisions of paragraphs (b), (c), (d), or (e) of this section.

(b) For certain drug products, the in vivo bioavailability or bioequivalence of the drug product may be self-evident. FDA shall waive the requirement for the submission of evidence obtained in vivo measuring the bioavailability or demonstrating the bioequivalence of these drug products. A drug product's in vivo bioavailability or bioequivalence may be considered self-evident based on other data in the application if the product meets one of the following criteria:

- (1) The drug product:
 - (i) Is a parenteral solution intended solely for administration by injection, or an ophthalmic or otic solution; and
 - (ii) Contains the same active and inactive ingredients in the same concentration as a drug product that is the subject of an approved full new drug application or abbreviated new drug application.
- (2) The drug product:

- (i) Is administered by inhalation as a gas, e.g., a methonal or an inhalation anesthetic; and

- (ii) Contains an active ingredient in the same dosage form as a drug product that is the subject of an approved full new drug application or abbreviated new drug application.

- (3) The drug product:

- (i) Is a solution for application to the skin, an oral solution, elixir, syrup, tincture, a solution for aerosolization or nebulization, a nasal solution, or similar other solubilized form; and

- (ii) Contains an active drug ingredient in the same concentration and dosage form as a drug product that is the subject of an approved full new drug application or abbreviated new drug application; and

- (iii) Contains no inactive ingredient or other change in formulation from the drug product that is the subject of the approved full new drug application or abbreviated new drug application that may significantly affect absorption of the active drug ingredient or active moiety for products that are systemically absorbed, or that may significantly affect systemic or local availability for products intended to act locally.

- (c) FDA shall waive the requirement for the submission of evidence measuring the in vivo bioavailability or demonstrating the in vivo bioequivalence of a solid oral dosage form (other than a delayed release or extended release dosage form) of a drug product determined to be effective for at least one indication in a Drug Efficacy Study Implementation notice or which is identical, related, or similar to such a drug product under § 302.6 of this chapter unless FDA has evaluated the drug product under the criteria set forth in § 320.33. Included the drug product in the Approved Drug Products with Therapeutic Equivalence Evaluations List, and rated the drug product as having a known or potential bioequivalence problem. A drug product so rated reflects a determination by FDA that an in vivo bioequivalence study is required.

- (d) For certain drug products, bioavailability may be measured or bioequivalence may be demonstrated by evidence obtained in vitro in lieu of in

Pharmacokinetics and Hematological Effects of the PEGylated Thrombopoietin Peptide Mimetic GW395058 in Rats and Monkeys After Intravenous or Subcutaneous Administration

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Key Words: Thrombopoietin; Mimetic peptide; GW395058; Pharmacokinetics; Hematology; Rat; Monkey; PEGylated

ABSTRACT

GW395058, a potent PEGylated peptide human thrombopoietin receptor (HuTPOr) agonist *in vitro*, is being evaluated for the treatment of thrombocytopenia. GW395058 shares no sequence homology with TPO. In this report the pharmacokinetics and hematological effects of GW395058 in rats and monkeys are described. Doses eliciting thrombocytosis in rodents (2 or 10 µg/kg s.c.) produced insufficient plasma concentration data for pharmacokinetic parameter estimate calculations. At higher i.v. doses in rats (500, 1,000 or 2,000 µg/kg) serum *t*_{1/2} (half-life) values were >20 h, and the area under the concentration time curve increased proportionally with dose. In cynomolgus monkeys GW395058 plasma *t*_{1/2} values ranged from 37 to 68 h after s.c. or i.v.

dosing, and similar values were observed in rhesus monkeys following s.c. dosing. Rat platelet counts increased following 2 (1.6-fold) or 10 µg/kg (fourfold) s.c. doses. Cynomolgus and rhesus monkey platelet counts did not change significantly at comparable s.c. doses, but did increase slightly (<twofold) in cynomolgus monkeys following a 25 µg/kg s.c. dose and twofold following a 100 µg/kg s.c. dose. Because the plasma *t*_{1/2} of GW395058 is long in mouse, rat, dog, and monkey, yet the dose required to double platelet levels in monkeys is 50-400 that required in rats, differences in hematological responses may be due to interspecies differences in the interaction of GW395058 with the TPOr. *Stem Cells* 1999;17:316-326

INTRODUCTION

Neutropenia and thrombocytopenia are chemotherapy-induced side effects associated with significant morbidity, including spontaneous bleeding. Thrombocytopenia often limits the maximum chemotherapy dose and the dosing regimen that can be administered. Reducing the magnitude of thrombocytopenia might allow administration of higher doses of chemotherapeutic agents or dose-intensification, which could result in improved disease control. Standard practice is to manage thrombocytopenia by administering platelet transfusions, which carry a risk of secondary infection as well as the eventual production of neutralizing antibodies to platelets. Recombinant interleukin 11 (IL-11) has

been approved for the treatment of chemotherapy-induced thrombocytopenia, although its use has been associated with notable side effects [1-3].

The lineage-specific cytokine that regulates platelet production, thrombopoietin (TPO), has been described [4]. Two forms of the recombinant human protein have been investigated: full-length glycosylated recombinant human protein (rhTPO) [5] and PEGylated megakaryocyte growth and development factor (PEG-rHuMGDF) [6]. In mice, dogs, and primates [7-14], rhTPO and PEG-rHuMGDF promote platelet production and reduce chemotherapy-induced thrombocytopenia. Unfortunately, evidence of TPO-neutralizing antibodies in patients participating in cancer and

platelet donor clinical trials forced the discontinuation of PEG-rHuMGDF development [15].

Not long after TPO was first reported, a 28-amino acid TPO mimetic peptide was described that was equipotent to TPO in cell-based assays [16]. This peptide dimer, AF13948, shares no sequence homology with TPO. Studies showed that while AF13948 plasma concentrations were short in mice (plasma *t*_{1/2} [half-life] = 1 h), AF13948 stimulated platelet production following single or multidose regimens of 250 or 1,000 µg/kg (unpublished results).

Subsequent to these studies the sequence of AF13948 was modified with amino acid substitution, and the resulting peptide, AF15705, was PEGylated to produce GW395058 (Fig. 1). Similar to AF13948 and AF15705, GW395058 binds to and activates the human TPO receptor (HuTPOr) with an affinity similar to that of TPO *in vitro* [17]. Unlike AF13948, GW395058 has a long *t*_{1/2} in mice and elevates peripheral platelet counts following a single s.c. dose of as little as 2 µg/kg [18]. In dogs, when coadministered with G-CSF, GW395058 has a long plasma half-life and accelerates platelet recovery in a dog myelosuppression model (unpublished results, manuscript in preparation). Recent studies have shown that GW395058 is not immunogenic in several animal species, and that if an antibody response to GW395058 were elicited, those antibodies would not likely neutralize endogenous HuTPO [19].

In this report, we describe the pharmacokinetics and hematological effects of GW395058 in rats and monkeys following i.v. or s.c. administration of the compound.

EXPERIMENTAL PROCEDURES

Materials

AF15705 and GW395058 were obtained from the Biotechnology Product Development Division, Glaxo Wellcome Inc. (Research Triangle Park, NC). All other chemicals were reagent grade or better. Dosing solutions of GW395058 were prepared in phosphate-buffered saline (PBS). GW395058 was prepared by derivatizing the two N-termini of the AF15705 linear dimer with 20,000 molecular weight (mw) polyethylene glycol (PEG) (unpublished results, manuscript in preparation). The mw of AF15705 and GW395058 are 3,295 and ~43,300, respectively. All dose calculations are based on peptide mass and are thus directly comparable for PEGylated and unmodified peptides. The amino acid sequences of AF15705 and GW395058 are shown in Figure 1.

Animal Handling, Dosing, and Sample Collection

All animal procedures described in this report were approved by the Institutional Animal Care and Use Committee and conducted in accordance with Federal guidelines.

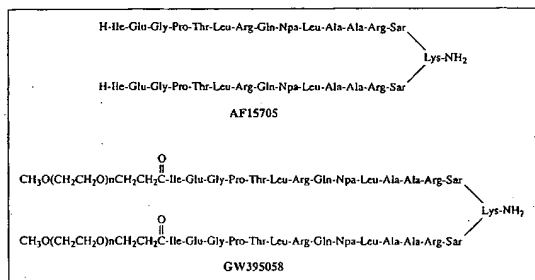


Figure 1. Amino acid sequences of AF15705 and GW395058. AF15705 is a peptide dimer consisting of two identical amino acid chains of 14 residues linked through their carboxyl termini to the α and ϵ amino groups of a lysine residue. The sequence of AF13948 (see text) was modified by the replacement of an alanine and a β -alanine residue with sarcosine (Sar) to remove the chiral centers. Tyrosine residues were replaced with naphthylalanine (Npa) for ease of synthesis. The two resulting peptide amino termini of AF15705 were conjugated to 20,000 mw PEG to produce GW395058.

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Studies in Rats

Single-dose and one-month repeat-dose studies were conducted in Han Wistar rats at Glaxo Wellcome. In the single-dose study, male rats (300 to 350 g, three rats/dose) received single s.c. doses of 2 or 10 µg/kg. Plasma samples for determination of GW395058 concentrations were obtained from each rat predose and at 3, 4, 7, and 10 days postdose. Plasma samples were stored at -70°C until analysis. Blood samples for hematological analysis were obtained from each rat predose and at 3, 7, 10, 14, 17, 21, and 28 days postdosing and analyzed the same day.

In the one-month repeat-dose study, male and female Han Wistar rats (250 to 300 g; 10 rats/sex at the 500 and 1,000 µg/kg/48 h doses, 18 rats/sex at the control and the 2,000 µg/kg/48 h doses) received single i.v. bolus doses every other day for a month. Upon completion of dosing, 10 rats/sex/dose were euthanized, while remaining control and high-dose rats were allowed to recover for one month and then euthanized to assess the reversibility of treatment-related effects. Doses of GW395058 in this study were chosen to provide exposures well above those necessary for efficacy in rats and those anticipated in clinical trials. Following doses 1 and 11, serum samples for determination of GW395058 concentration were obtained from 3 rats/sex/time point predose and at 0.5, 0.5, 1, 2, 4, 8, 24 and 48 h after dosing. Following dose 11, additional serum samples were taken at 72 and 96 h. Serum samples were stored at -70°C until analysis. Blood samples for hematological analysis were obtained from each rat predose, on day 24 (near the end of the dosing period), and 13 and 28 days after dosing stopped. Hematologic samples were analyzed the same day they were taken.

Single-dose and one-month repeat-dose studies were conducted in monkeys at Covance (Vienna, VA). In the single-dose study, male cynomolgus monkeys (3 to 5 kg) received single s.c. doses at 2, 10, 25, or 100 µg/kg (three monkeys/dose) or a single i.v. dose at 10 µg/kg (two monkeys). Plasma samples for pharmacokinetic analysis were obtained from each monkey predose and at 0.08, 0.25, 0.5, 1, 2, 4, 8, 24, 72, 96, 120, 144, and 168 h postdosing. Plasma samples were stored at -70°C until analysis. Blood samples for hematological analysis were obtained from each monkey at predose (day 1) and 3, 5, 7, 8, 10, 11, 12, 15, 17, 19, and 22 days postdose. Blood samples were stored at room temperature until processed (within 1 h of collection). Because platelet counts increased only slightly at 25 and 100 µg/kg and not at all at 510 µg/kg, four rhesus monkeys (3 to 5 kg; two males) were added to the study to determine if they responded more strongly to GW395058 than did cynomolgus monkeys. Rhesus monkeys received single s.c. doses of GW395058 at 2 or 10 µg/kg. Samples (plasma and blood) were collected as described for cynomolgus monkeys.

In the one-month repeat-dose study, male and female cynomolgus monkeys (3 to 5 kg; three/sex at 500 and 1,000 µg/kg/48 h doses, five/sex at control and at the 2,000 µg/kg/48 h dose) received single i.v. doses every other day for a month. Upon completion of dosing, three monkeys/sex/dose were euthanized, while remaining control and high-dose monkeys were allowed to recover for one month and then euthanized to assess the reversibility of treatment-related effects. Following doses 1 and 14, serum samples were obtained predose and at 0.08, 0.5, 1, 2, 4, 8, and 24 h postdose. Following the first dose, an additional serum sample was collected at 48 h from all animals. Following dose number 14, additional serum samples were taken from animals (two animals/sex) in the vehicle control and 2,000 µg/kg/48 h groups at 48, 72 and 96 h after dosing. Serum samples were stored frozen at or below -20°C until analysis. Blood samples for hematological analysis were obtained from each monkey predose, on days 14 and 26 during the dosing period, and 10 and 24 days after dosing stopped. Hematologic samples were analyzed the same day they were taken.

Determination of GW395058 Plasma Concentrations

Radioimmunoassay (RIA)

Concentrations of GW395058-like immunoreactive material in rat and monkey plasma samples were determined by using a competitive binding RIA. A biotinylated analog of AF15705 was prepared by derivatizing the two N-termini of AF15705 with succinimidyl propionate. N¹-propionate-2,3-³H obtained from Dupont Company NEN Research Products (Boston, MA). The reaction product was purified by RP-HPLC (2.5 × 150 mm Symmetry® C18 5 µm column, Waters Corp. [Milford, MA]; mobile phase A = 0.1% Trifluoroacetic acid [TFA] in water; mobile phase B = 0.1% TFA in acetonitrile) by using gradient chromatographic conditions (0 to 60% mobile phase B for 30 min followed by 60% to 75% mobile phase B for 5 min). The purified radioligand had a specific activity of ~25Ci/nmol.

In this RIA diluted rabbit antiserum [19] raised to a bovine thyroglobulin conjugate of an AF15705-analog was incubated overnight at 4°C with radioligand. (H-AF15705) and aliquots of study samples or spiked (GW395058) plasma calibration standards. The next day bound and free radioligands were separated by incubating for 1 h at 4°C with anti-rabbit immunoprecipitation reagent (Sic-Cat®; IDS Ltd.; Boldon, Tyne and Wear, UK), followed by the addition of 2 ml of cold PBS (pH 7.2) containing 0.1% bovine serum albumin and 0.1% Tween-20. Assay tubes were centrifuged for 10 min and the supernatant aspirated to waste. The pellets were resuspended in 2 ml of water and decanted into 20 ml scintillation vials containing 5 ml of scintillation fluid. Bound

radioligand was quantified in a liquid scintillation counter, and calibration curves were derived by using the program Multistate (Wallac; Turku, Finland), from which control and study sample concentrations were interpolated. All samples were assayed in duplicate. The lower limit of quantitation (LLOQ) was set at 1.6 ng/ml. Because this RIA may also detect metabolic fragments of GW395058, the results here are reported as GW395058 immunoreactivity equivalents.

TPOR Biological Activity Assay

Concentrations of GW395058-like biologically active material in monkey plasma samples following a 25 µg/kg s.c. dose of GW395058 were determined by using a reporter assay based on a Baf3 HuTPOR fos/lux cell line. In these cells activation of the HuTPOR results in the production of luciferase that catalyzes the emission of light upon addition of the substrate luciferin. The cells were grown in Dulbecco's modified Eagle's essential medium/F12, 10% fetal bovine serum; 10% Wehi-3 supernatant and antibiotics/antimycotics. Approximately 18 h before the assay, cells were starved by transferring them to the same medium made 0.1% Wehi-3 supernatant. On the day of assay, following a washing step in medium without Wehi-3, 1 × 10⁶ cells/ml were combined with study samples or GW395058-spiked plasma calibration standards. Assay plates were incubated for 2 h at 37°C in a 5% CO₂ atmosphere, then Lucite reagent (Packard Instrument Co.; Downers Grove, Ill.) was added to each well. Light emission was measured after a five-minute incubation on a Packard Topcount Lumiscimeter (Packard Instrument Co.). The concentrations of GW395058-like biological activity in the plasma samples were interpolated from the linear portion of the standard curve. The LLOQ was set at 0.1 ng/ml. Because this assay does not distinguish GW395058 from other possible agonists, results with this method were reported as GW395058 biological activity equivalents.

Pharmacokinetic Calculations

Area under the concentration time curve (AUC) values for GW395058 were estimated by using the trapezoidal rule, with back-extrapolation of the GW395058 plasma concentrations to zero time. Single day 1 AUC values were calculated from time zero to infinity (0-∞), with the extrapolated area from the time of the last measurable sample concentration (C_{last}) to infinity time estimate as C_{last} × t_{1/2}, where t_{1/2} is the terminal phase elimination rate constant. Multidose day last AUC values were calculated from time zero to t, where t is the dosing interval. C_{max} values were defined as the highest observed plasma concentration postdose. Mean calculations were performed by using Microsoft® Excel 97 SR-1 (Microsoft Corporation; Redmond, WA). Noncompartmental pharmacokinetic calculations were performed by using WinNonlin, Professional Network Version 1.5 (Scientific

Consulting Inc.; Cary, NC) or PKCAL Version 2.0 (Glaxo Wellcome Inc.).

Hematology

Clinical hematology and coagulation analyses were performed at Glaxo Wellcome (rats) or at Covance, Vienna, Virginia (monkeys). Clinical hematology parameters measured in all studies were platelet count, total leukocyte count, differential leukocyte counts, total erythrocyte count, reticulocyte count, hematocrit, hemoglobin, and RBC indices (mean cell hemoglobin [MCH], mean cell volume [MCV], mean cell hemoglobin concentration [MCHC]). In one-month studies, mean platelet volume (MPV), platelet distribution width, and coagulation parameters (prothrombin time and activated partial thromboplastin time) also were measured.

Statistical Analysis

Mean calculations were performed using Microsoft® Excel 97 SR-1. Statistical analysis (ANOVA) of pharmacokinetic and hematology data was performed by using JMP Statistical Visualization Software Version 3.2.5 (SAS Institute, Inc.; Cary, NC) or SAS System for Windows, Version 6.12, TS Level 0020, (SAS Institute). Comparisons of pharmacokinetic parameter estimates were made between the sexes and days of dosing and among the dose groups by using Tukey's Studentized Range Test. Differences were declared statistically significant when p < 0.05.

RESULTS

Comparison of RIA with TPOR Biological Activity Assay

To clarify the relationship between GW395058 immunoreactivity and TPOR biological activity, plasma concentrations of GW395058 were measured by RIA and TPOR biological activity assay in samples collected following a single s.c. dose of GW395058 at 25 µg/kg to male cynomolgus monkeys (three/group). Averaged plasma concentrations of GW395058 determined by RIA or TPOR biological activity assay are shown in Table 1. A comparison of individual GW395058 plasma concentrations measured by RIA or TPOR biological activity assay is shown in Figure 2.

Good general agreement (r² = 0.95; slope = 0.74) was found between the TPOR biological activity assay and the GW395058 RIA. The results show that the RIA detects primarily biologically active material in plasma, and this assay can be used to accurately determine GW395058 plasma or serum concentrations in monkeys or rats dosed with GW395058. Although the TPOR biological activity assay was the slightly more sensitive assay of the two methods, the RIA had a greater throughput. Consequently, the RIA was used to determine GW395058 plasma or serum concentrations for calculation of pharmacokinetic parameter estimates in study samples.

39

Time (h)			TPOR biological activity assay		
RIA			TPOR biological activity assay		
(ng/ml GW395058 equivalents ± SD)			(ng/ml GW395058 equivalents ± SD)		
Protein			Protein		
NQ			NQ		
0.05	NQ		0.05	NQ	
0.25	1.8 ± 0.5		0.25	2.0 ± 1.5	
0.5	6.7 ± 1.4		0.5	6.9 ± 0.6	
1	21 ± 14		1	20 ± 12	
2	42 ± 28		2	45 ± 24	
4	107 ± 45		4	90 ± 31	
8	130 ± 23		8	107 ± 27	
24	159 ± 31		24	127 ± 16	
48	141 ± 52		48	109 ± 18	
72	119 ± 37		72	87 ± 20	
96	86 ± 27		96	61 ± 15	
120	64 ± 28		120	49 ± 15	
144	45 ± 21		144	33 ± 13	
168	29 ± 11		168	25 ± 12	

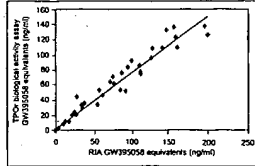


Figure 2. Comparison of GW395058 plasma concentrations determined by RIA or TPOR biological activity assay. Individual concentrations (GW395058 equivalents ng/ml plasma) determined by RIA or TPOR biological activity assay are shown. Plasma samples were obtained from male cynomolgus monkeys (three/group) receiving a single s.c. dose at 25 µg/kg.

Plasma Concentrations and Pharmacokinetic Parameter Estimates for GW395058 in Han Wistar Rats

Following s.c. dosing with GW395058 at 2 or 10 µg/kg, there were insufficient plasma concentration versus time data

available to estimate pharmacokinetic parameters due to the limited amount of blood and plasma that could be collected from each animal over the extended duration of this study. The highest plasma concentrations of GW395058 were observed on day 3 following the 10 µg/kg dose. GW395058 was undetectable in plasma following the 2 µg/kg dose (data not shown).

Pharmacokinetic parameter estimates for GW395058 in male and female Han Wistar rats following i.v. dosing at 500, 1,000, or 2,000 µg/kg/48 h are shown in Table 2.

In general following i.v. dosing at 500, 1,000, or 2,000 µg/kg/48 h for one month, plasma concentrations did not differ between sexes. Following dose 1, there was good proportionality with increasing dose between AUC and Cmax values. Plasma t_{1/2} values were long at all dose levels. Plasma clearance (CL) and volume of distribution values (V_d) over the fourfold range in i.v. dose were low and generally comparable among dose levels. Dose 1 and dose 11 pharmacokinetic parameter estimates did not differ (data not shown) suggesting that repeated i.v. dosing did not alter GW395058 systemic exposure.

Plasma Concentrations and Pharmacokinetic Parameter Estimates for GW395058 in Cynomolgus Monkeys

Plasma concentrations of GW395058 in cynomolgus monkeys following s.c. dosing at 2, 10, 25, and 100 µg/kg and i.v. at 10 µg/kg are shown in Figure 3. Pharmacokinetic parameter estimates for GW395058 in cynomolgus monkeys following s.c. dosing at 2, 10, 25, and 100 µg/kg and i.v. at 10 µg/kg are shown in Table 3.

In general, AUC and Cmax values increased proportionally with increasing dose following s.c. dosing at 2, 10, 25, or 100 µg/kg. Plasma t_{1/2} values were long at all dose levels. Plasma clearance (CL/F) values over the 50-fold range in s.c. dose were low and comparable among dose levels. Volume of distribution (V_d/F) values were similar at the 10, 25, and 100 µg/kg dose levels, while the averaged V_d/F value for the 2 µg/kg dose was slightly higher.

Following i.v. dosing with GW395058 at 10 µg/kg, the AUC was slightly lower than the comparable s.c. dose level. The plasma t_{1/2} following i.v. dosing was at least 10 h less than that observed following s.c. dosing. Plasma CL following i.v. dosing was generally low, but slightly higher than that observed following s.c. dosing at the same level. The V_d following i.v. dosing at 10 µg/kg was essentially the same as that observed following s.c. dosing. The volume at steady state (V_{ss}) was 82 ± 12 mL/kg.

Pharmacokinetic parameter estimates for GW395058 in cynomolgus monkeys following single and repeated i.v. doses at 500, 1,000, 2,000 µg/kg are shown in Table 4. In general for monkeys (male and female) dosed at 500, 1,000,

Average Pharmacokinetic Parameter Estimates						
Sex	Dose (µg/kg/48h)	AUC ₀₋₄₈ (h * ng/ml)	Cmax (ng/ml)	t _{1/2} (h)	CL (ml/h/kg)	V _d (ml/kg)
M	500	219,254	10,426	21.6	2.28	71.0
F	500	290,411	15,145	30.2	1.72	75.1
M	1,000	565,297	30,061	23.0	1.77	58.7
F	1,000	618,189	27,017	20.4	1.62	47.6
M	2,000	983,877	54,947	18.2	2.03	51.6
F	2,000	1,120,452	58,904	19.4	1.77	49.1

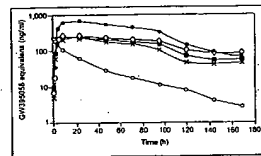


Figure 3. Plasma concentrations in male cynomolgus monkeys dosed with GW395058. Average plasma concentrations (GW395058 equivalents ng/ml plasma) determined by RIA are shown. Plasma samples were obtained from monkeys (three/group) receiving single s.c. doses at 2 (Δ), 10 (□), 25 (○), or 100 (●) µg/kg or on i.v. dose at 10 (◇) µg/kg.

or 2,000 µg/kg; A) Cmax and AUC values increased proportionally with dose; B) t_{1/2} values were long, and C) CL values were low and comparable among dose levels.

Following doses 1 and 14, Cmax and AUC values for GW395058 increased with increasing doses. Increases in Cmax and AUC values between 500 and 2,000 µg/kg groups were statistically significant. The increases in CL between

500 and 2,000 µg/kg dose groups were small, and only significant in female monkeys. There were no statistically significant differences in t_{1/2} or V_d across the dose groups.

Cmax values increased from dose 1 to dose 14, but these increases were significant only in the 1,000 and 2,000 µg/kg female and the 500 and 1,000 µg/kg male dose groups. The only significant decrease in CL and increase in AUC values following multiple dosing were observed in female monkeys at the 2,000 µg/kg dose. There were no significant changes in t_{1/2} or V_d with multiple dosing.

Cmax, AUC, t_{1/2}, and CL values following doses 1 and 14 were not statistically different between male and female monkeys, except at the 2,000 µg/kg dose group, where Cmax, AUC, and t_{1/2} were greater in males and CL was greater in females. There were no other sex-related trends in any of the other pharmacokinetic parameters examined.

Plasma Concentrations and Pharmacokinetic Parameter Estimates for GW395058 in Rhesus Monkeys

Pharmacokinetic parameter estimates for GW395058 in the rhesus monkey following s.c. dosing at 2 and 10 µg/kg are shown in Table 5. Pharmacokinetic parameter estimates in rhesus monkeys were comparable to those for cynomolgus monkeys following s.c. dosing at 2 and 10 µg/kg. AUC values

Pharmacokinetic Parameter Estimates (Average ± SD)						
Route	N	Dose (µg/kg)	AUC ₀₋₂₄ (h * ng/ml)	Cmax (ng/ml)	t _{1/2} (h)	V _d (ml/kg)
i.v.	2	10	5,134	236	37	103
s.c.	3	2	1,367	13	68	145
s.c.	3	10	7,217	71	47	95
s.c.	3	25	18,054	161	45	94
s.c.	3	100	65,863	704	45	104

Averaged Pharmacokinetic Parameter Estimates Following Dose 1 and 14							
Dose #	Sex	Dose (µg/kg/48h)	AUC _{0-∞} (h · ng/ml)	C _{max} (ng/ml)	T _{1/2} (h)	CL (ml/kg)	V _d (ml/kg)
1	M	500	528,006	18,493	26.1	0.95	33.9
	F	500	446,043	15,221	23.4	1.17	40.6
	M	1,000	703,681	32,572	23.4	1.51	49.8
	F	1,000	776,712	30,441	23.7	1.31	43.9
	M	2,000	1,038,371	57,915	33.8	1.15	55.0
	F	2,000	1,094,825	42,009	24.2	1.89	65.4
14	M	500	633,845	17,893	27.2	0.82	30.9
	F	500	568,493	21,137	32.7	0.92	41.3
	M	1,000	1,092,922	44,004	44.0	0.97	53.6
	F	1,000	923,744	38,889	25.1	1.08	39.1
	M	2,000	1,540,563	64,384	39.3	1.37	72.1
	F	2,000	1,468,930	65,525	31.3	1.38	62.6

*AUC_{0-∞} for dose 1, AUC₀₋₁₄ for dose 14.

Pharmacokinetic Parameter Estimates (Average ± SD)						
Dose (mg/kg)	AUC _{0-∞} (h · ng/ml)	C _{max} (ng/ml)	T _{max} (h)	T _{1/2} (h)	CL/F (ml/kg)	V _d /F (ml/kg)
2	1,002	15	8	39	1.9	78
10	5,710	69	16	45	1.8	113

increased proportionally with increasing dose for rhesus monkeys following s.c. dosing at 2 and 10 µg/kg. Similarly, C_{max} values were comparable with those at the same dose levels for cynomolgus monkeys (Table 3), and there was proportionality with increasing dose. T_{max} values for rhesus monkeys were slightly shorter than those for cynomolgus monkeys. Plasma t_{1/2} values for rhesus monkeys were long following s.c. dosing, although the plasma t_{1/2} value at the 2 µg/kg dose level was approximately half that observed for cynomolgus monkeys. CL/F and V_d/F values were similar for both rhesus and cynomolgus monkeys at the 2 and 10 µg/kg dose levels.

Hematological Effects in Rats After Single-Dose Studies

Single s.c. doses of GW395058 at 2 or 10 µg/kg altered platelet, RBC, and WBC parameters. Platelet counts were increased two- to fourfold within three days postdose and returned to normal within 14 days postdose. The platelet response was dose-dependent, in that increases occurred more rapidly, persisted longer, and were greater in magnitude at

10 µg/kg (Fig. 4). RBC parameters (hematocrit, hemoglobin, RBC count) decreased slightly (10%-15%) at both doses

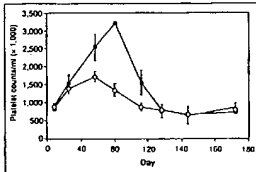


Figure 4. Platelet counts in male Han-Wistar rats dosed with GW395058. Average platelet counts in rats are shown. Blood samples were obtained from rats (three/group) receiving single s.c. doses of GW395058 at 2 (○) or 10 (●) µg/kg.

within three days postdose, and did not return to normal until 28 days postdose. Decreases occurred more rapidly and were greater in magnitude at 10 µg/kg. There was a corresponding increase in red cell distribution width, which remained elevated for 28 days postdose, the last time point measured (data not shown).

Total WBC counts increased slightly (twofold) at both doses within three days postdose and returned to normal within 14 days postdose, reflecting an across-the-board increase in lymphocyte and granulocyte counts. Increases in WBC counts were unrelated to dose (data not shown).

Hematological Effects in Rats After One-Month Repeat-Dose Studies

Repeated i.v. administration of GW395058 at 500, 1,000, or 2,000 µg/kg every other day for a month produced much greater changes in platelet, RBC, and WBC parameters than those observed after single low doses. However, at these elevated dose levels, hematologic effects did not differ among dose groups (Table 6). Platelet counts increased about threefold during the dosing period but increased more (about 10-fold) within two weeks after dosing stopped. Platelet counts had returned to normal by a month postdose. Increases in platelet count were accompanied by an increase in MPV.

RBC parameters (hematocrit, hemoglobin, and RBC count) decreased 15%-25% by the end of the dosing period, and returned to normal within a month postdose. Decreases were accompanied by a reduction in mean corpuscular hemoglobin concentration (5%-10%) and an increased red cell distribution width (15%-30%).

Total WBC counts increased markedly (about eightfold) by the end of the dosing period and returned to normal within a month postdose. WBC count increases reflected across-the-board increases in lymphocyte counts (about fourfold) and granulocyte counts (about 20-fold) observed during this study.

Prothrombin time was somewhat prolonged (30%) at the end of the dosing period, but returned to normal within a month postdose. Activated partial thromboplastin time was unaltered (data not shown).

Hematological Effects in Monkeys After Single-Dose Studies

In cynomolgus or rhesus monkeys, no significant changes in platelet counts were observed following s.c. doses at 2 or 10 µg/kg. In cynomolgus monkeys, a 25 µg/kg s.c. dose produced small but significant increases in platelet counts on days 7 and 11 (1.3 times and 1.5 times, respectively) from day 1 values (data not shown). A 100 µg/kg s.c. dose produced significant increases in platelet counts on days 9, 11, and 13

Dose (µg/kg/48 h)	Sample time				
	0	500	1,000	2,000	2,000
Platelet effects					
Platelet count (x10 ⁹ /mm ³)	1,504	2,262	2,243	2,304	6,796
MPV (µ ³)	5.1	6.0	6.1	6.1	5.4
Granulocyte effects					
Neutrophil count (/mm ³)	941	19,802	18,870	27,439	22,330
Monocyte count (/mm ³)	160	7,917	6,790	8,246	7,384
Eosinophil count (/mm ³)	86	1,067	810	926	1,355
Basophil count (/mm ³)	11	653	329	861	290
Lymphocyte effects					
Lymphocyte count (/mm ³)	4,210	24,795	24,874	27,439	41,620
RBC effects					
RBC count (x10 ¹² /mm ³)	7.05	5.56	5.53	5.52	5.46
RDW (%)	12.7	18.7	18.7	18.0	19.8
Coagulation effects					
Prothrombin time (sec)	13.7	16.6	17.2	17.5	n.d.

n.d. = not determined; RDW = red cell distribution width

(Fig. 5). However, platelet counts did not increase proportionally with dose from 25 to 100 µg/kg. Additionally, cynomolgus monkeys dosed at 100 µg/kg had significant increases in total WBC counts (1.4-fold) on day 7 (data not shown) and neutrophil counts on day 7 (2.6 times) from day 1 counts (Fig. 6). No significant changes were observed for the other hematological parameters examined.

Hematological Effects in Monkeys After One-Month Repeat-Dose Studies

Repeated i.v. administration of GW395058 to cynomolgus monkeys at 500, 1,000, and 2,000 µg/kg/48 h for a month produced greater increases in platelet counts than were observed in single-dose monkey studies. In addition, RBC and WBC parameters were altered. However, neither the extent nor magnitude of hematological effects differed among dose groups (Table 7). RBC parameters were unaffected

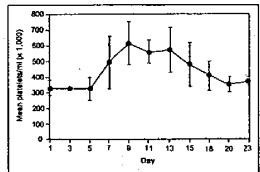


Figure 5. Platelet counts in male cynomolgus monkeys dosed with GW395058. Average platelet counts in monkeys are shown. Blood samples were obtained from monkeys (three/group) receiving a single s.c. dose of GW395058 at 100 µg/kg.

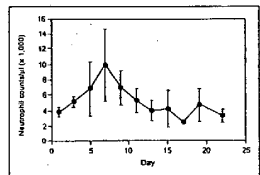


Figure 6. Neutrophil counts in male cynomolgus monkeys dosed with GW395058. Average neutrophil counts in monkeys are shown. Blood samples were obtained from monkeys (three/group) receiving a single s.c. dose of GW395058 at 100 µg/kg.

during the dosing period, but a mild transient regenerative anemia occurred postdose. This was characterized by slightly decreased RBC parameters (RBC count, hematoctrit, hemoglobin) and increased red cell distribution width. Anemia had not completely resolved a month after dosing stopped, although RBC parameters approached normal values.

Total WBC counts increased slightly (two-fold) by the end of the dosing period and remained slightly increased for a month postdose. WBC count increases reflected an across-the-board increase in lymphocyte counts (about twofold) and granulocyte counts (neutrophils twofold, monocytes and eosinophils about fourfold) observed in this study.

Platelet counts increased about sevenfold during the dosing period and returned to approximately normal levels within a month postdose. Increases in platelet counts were accompanied by an increased platelet distribution width and a decreased MPV.

Activated partial thromboplastin time was somewhat prolonged (10%) at the end of the dosing period, but returned to normal within a month postdose. Prothrombin time was unaltered (data not shown).

DISCUSSION

This report shows that in rats (i.v.), or cynomolgus (i.v. or s.c.), or rhesus (s.c.) monkeys, administration of GW395058 results in dose-proportional exposures with the peptide exhibiting a low CL/F and V_d, and a long plasma t_{1/2}. The pharmacokinetic behavior of GW395058 is similar in cynomolgus and rhesus monkeys. While repeated administration of GW395058 for a month at high doses stimulated megakaryocytopoiesis, granulocytopoiesis, and lymphocytopoiesis in both rats and cynomolgus monkeys, the nature and magnitude of the responses differed between species. Following repeated administration of GW395058, platelet counts increased more in monkeys than rats, while WBC counts increased more in rats than monkeys. Platelet count increases were accompanied by a decrease in average platelet size in monkeys but an increase in average platelet size in rats. These differences may reflect species differences in receptor sensitivity or inherent responses to receptor stimulation.

Hematologic effects produced by repeated high doses of GW395058 were reversible when dosing stopped, although recovery was prolonged in some cases. This prolonged recovery phase may reflect GW395058's long t_{1/2}, but it also may reflect the inherent rate at which altered hematopoietic cell counts return to homeostatic levels in the species tested.

The observation that hematological effects in repeat-dose studies did not differ with dose suggests that the dose regimen used was beyond that needed to elicit a maximum response. This in turn suggests that maximum responses

Dose (µg/kg/48 h) =	Sample time				+ 2 weeks postdose	+ 4 weeks postdose
	0	500	1,000	2,000	2,000	2,000
Platelet effects						
Platelet count (x10 ⁹ /mm ³)	381	1,811	2,820	2,405	2,141	692
PDW (%)	40.6	59.1	56.2	57.0	53.9	49.4
MPV (µ ³)	9.4	6.9	7.1	7.3	8.1	9.4
Granulocyte effects						
Neutrophil count (/mm ³)	4,400	6,900	7,300	5,700	4,400	3,200
Monocyte count (/mm ³)	450	1,150	1,550	1,550	2,000	830
Eosinophil count (/mm ³)	150	450	800	350	400	400
Lymphocyte effects						
Lymphocyte count (/mm ³)	5,900	9,400	8,100	11,300	14,000	10,800
RBC effects						
RBC count (x10 ¹² /mm ³)	5.96	5.53	6.16	5.87	5.10	5.56
RDW (%)	14.8	15.4	15.7	15.4	17.5	16.7
Coagulation effects						
APT time (sec)	19.8	21.2	21.7	23.3	n.d.	n.d.

n.d. = not determined; PDW = platelet distribution width; RDW = red cell distribution width.

could be obtained with lower and/or less frequent doses. The dose regimen chosen made it impossible to characterize the dose-response curves for individual hematologic effects, so it is not possible to address the question here of whether the various effects were mediated by a similar molecular mechanism (i.e., action of GW395058 on a single receptor). GW395058-induced thrombocytosis was not associated with hemorrhage or thrombosis, suggesting that induced platelets function normally, as has been shown with PEG-rHuMGDF [6, 13].

Because GW395058 produces increased numbers not only of circulating platelets that appear functionally normal, but also of circulating granulocytes and lymphocytes, the data presented here suggest that GW395058 has the potential to ameliorate damage or hasten recovery of multiple hematopoietic cell lines from chemotherapy-induced damage, as has been indicated for rHuTPO [20].

Although GW395058 has a long plasma t_{1/2} in mice [18], rats, dogs and monkeys, the single s.c. dose required to double circulating platelet levels in monkeys is 50-fold that required in rats. In previous studies [17] GW395058 and rHuTPO were shown to bind with similar affinity to murine BaF3 cells transduced with the HuTPO α and equipotent in stimulating megakaryocyte colony formation and megakaryocyte maturation in cells cultured from human bone marrow *in vitro*. Additional studies will be required to determine if the observed differences in hematological responses between rodents and monkeys are due to a significantly reduced affinity of the monkey TPO α for GW395058, relative to the human and rodent receptors.

ACKNOWLEDGMENT

We wish to thank Mr. Scott Hovis for his assistance with analysis of study samples, Mr. Michael R. Emptage for his assistance with statistical analysis of the data, and Mrs. Susan C. de Souza for her editorial assistance.

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