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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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Señor

SUPERINTENDENTE DE INDUSTRIA Y COMERCIO

DIVISION DE NUEVAS CREACIONES

E. _____ S. _____ D. _____

Re: PI.-F. HOFFMANN-LA ROCHE AG.-Solicitud de Patente de Invención en Fase Nacional PCT para "ANTICUERPOS CONTRA LA ANGIOPOYETINA 2 HUMANA".-Clase C07K 16/22.-Expediente número 13-206.276.-

1. Me refiero al auto dictado por ese Despacho notificado por fijación en lista de 26 de noviembre de 2013, así como a mi solicitud de plazo de 13 de febrero de 2014.

2. Con el fin de responder a las objeciones expuestas por el Señor Examinador bajo los acápites de reivindicaciones relativas a un uso o a un segundo uso, claridad, y nivel inventivo, me permito presentar los siguientes argumentos técnicos y las siguientes modificaciones en el capítulo reivindicatorio de la solicitud de patente en cuestión, por medio de lo cual se superan completamente dichas objeciones.

3. **Modificaciones en el capítulo reivindicatorio**

Inicialmente me permito señalar que como parte de la respuesta a las objeciones realizadas por ese Despacho, el solicitante ha decidido realizar las siguientes modificaciones en el capítulo reivindicatorio:

- Las reivindicaciones 1 – 5 corresponden a las presentadas originalmente;
- Las reivindicaciones 6 – 8 presentadas originalmente han sido eliminadas.
- La reivindicación 9 (Nueva reivindicación 6) tiene como base la reivindicación 9 actualmente presentada, en donde se han especificado las secuencias de cada uno de los CDRs de del dominio variable de cadena pesada y ligera. Esta modificación presenta sustento en las páginas 15 - 16 de la memoria descriptiva.

- La reivindicación 10 (nueva reivindicación 7) corresponde a la reivindicación 10 originalmente presentada.

- La reivindicación 11 (nueva reivindicación 8) tiene como base la reivindicación 11 originalmente presentada.

Con base en lo anterior me permito manifestar que las modificaciones realizadas en el capítulo reivindicatorio de ninguna manera amplían el alcance de la solicitud presentada originalmente, cumpliendo por lo tanto a cabalidad con las disposiciones de patentabilidad establecidas en el artículo 34 de la Decisión 486 de 2000.

4. **Reivindicaciones relativas a un uso o a un segundo uso**

De acuerdo con el señor Examinador, las reivindicaciones 6 a 8 no son patentables en virtud de los requisitos establecidos en los artículos 14 y 21 de la Decisión Andina 486 de 2000.

Atendiendo a lo anterior y de conformidad con lo mencionado en el numeral 3 del presente memorial, el solicitante ha decidido eliminar las reivindicaciones 6 a 8 actualmente presentadas, con lo cual se supera completamente la objeción realizada por el señor Examinador a éste respecto.

5. **Claridad del capítulo reivindicatorio**

Ahora bien, en respuesta a las objeciones de claridad realizadas por ese Despacho, atentamente me permito señalar lo siguiente:

a) El capítulo reivindicatorio ha sido drásticamente limitado a proteger un anticuerpo caracterizado específicamente por sus 6 CDR's, de tal manera que la nueva reivindicación 1 se lee como sigue:

"1. Un anticuerpo que se une específicamente a la angiopoyetina-2 humana (ANG-2), caracterizado porque:

a) el dominio variable de cadena pesada comprende una región CDR3 de la SEQ ID NO: 33, una región CDR2 de la SEQ ID NO: 34, y una región CDR1 de la SEQ ID NO: 35, y

b) el dominio variable de cadena ligera comprende una región CDR3 de la SEQ ID NO: 36, una región CDR2 de la SEQ ID NO: 37, y una región CDR1 de la SEQ ID NO: 38."

b) De lo anterior es claro que el resto de posibles secuencias de CDR's han sido eliminadas y por lo tanto dicho nuevo capítulo reivindicatorio es plenamente conciso;

c) La reivindicación 3 presentada originalmente ha sido eliminada, con lo cual se superan las objeciones realizadas a éste respecto;

d) La reivindicación 5 está dirigida a proteger una composición farmacéutica caracterizada porque comprende el anticuerpo revelado específicamente en la nueva reivindicación 1, descripción que permite a la persona medianamente versada en la materia determinar sin riesgo de confusión el alcance de dicha reivindicación; y,

e) La molécula de ácido nucleico y el vector reclamados en las nuevas reivindicaciones 6 y 7 se encuentran plenamente limitadas por las secuencias del anticuerpo definido en la nueva reivindicación 1, razón por la cual de ninguna manera le restan claridad a la solicitud en cuestión.

Finalmente y en vista de lo arriba mencionado me permito manifestar que el nuevo capítulo reivindicatorio de la solicitud de patente en cuestión (*el cual está compuesto por 8 reivindicaciones*) cumple a cabalidad con las disposiciones de patentabilidad establecidas en el Artículo 30 de la Decisión 486 de 2000, toda vez que es claro, conciso y encuentra pleno sustento en la descripción de la invención.

6. **Objeciones frente al nivel inventivo**

Finalmente, el señor Examinador objetó el nivel inventivo de la invención con base en las enseñanzas de los siguientes documentos: US 2006/0246071 (**D1**) y US 2006/0018909 (**D2**).

En relación con lo anterior y con el ánimo de demostrar que el nuevo capítulo reivindicatorio presenta un inequívoco carácter inventivo frente a la combinación de las enseñanzas de los documentos D1 y D2, me permito presentar ante ese Despacho las siguientes consideraciones técnicas:

Como se explicara en forma detallada más adelante, la persona normalmente versada en la materia hubiese considerado que dentro de los anticuerpos conocidos en el estado de la técnica, revelados por D1 y D2, el anticuerpo Mab536 como el más potente inhibidor de ANG-2, enlazando con su receptor Tie-2, convirtiéndose en el mejor candidato como agente terapéutico. Indistintamente el anticuerpo Ab3.19.3 el cual está siendo actualmente desarrollado y corresponde a una versión ligeramente

modificada en la región marco es denominado como MEDI3617, en donde las secuencias de los dominios VH y VL son conocidas en el artículo científico: *Buchanan, et al., mAbs 5 (2013) 255 – 262, Figura 1A*, (correspondiente al anexo III del presente memorial), las cuales son idénticas a las secuencias del anticuerpo MEDI 1/5 del documento WO2009/097325 (Párr. 18 – 19 y las Secuencias SEQ ID No 3 y 7, correspondiente al anexo V del presente memorial). En este sentido, el anticuerpo Ab 3.19.3 con las modificaciones mencionadas denominado MED3617, es desarrollado y se convierte en el anticuerpo preferido.

a) Con respecto al documento US 2006/0246071 (D1)

Por su parte, el documento D1 divulga básicamente una serie de anticuerpos monoclonales contra Angiopoyetina 2 (ANG-2), y sus valores IC_{50} para la unión de hANG-2 con Tie-2. De hecho, en la página 27 (párrafo [0208] y la Tabla 4 de D1 se establece específicamente que: *"el valor IC_{50} del "top 27" (D1) fue determinado, y aquellos anticuerpos preferidos "top 10" son listados en la Tabla 4"* (en éste punto considero de gran importancia resaltar que si bien el encabezado de la Tabla 4 hace referencia a valores EC_{50} , de la descripción es absolutamente claro que en realidad se divulgan valores IC_{50}).

Ahora bien, de acuerdo con los datos experimentales divulgados en D1, el anticuerpo más potente allí revelado, esto es, el clon 3.31.2, tiene un valor IC_{50} de 0,04169 $\mu\text{g/mL}$, valor que equivale a 0,28 nM teniendo en cuenta que un anticuerpo IgG tiene un peso molecular de 150 kD¹.

Dicho valor IC_{50} (0,28 nM) resulta ser entre dos y tres veces mayor que el valor IC_{50} del anticuerpo Ab 536 divulgado en el documento D2, hecho que demuestra que los anticuerpos divulgados en el documento D1 de ninguna manera representan el arte previo más cercano a la solicitud de patente en cuestión y por lo tanto no desvirtúan su nivel inventivo.

b) Con respecto al documento US 2006/0018909 (D2)

El documento D2 divulga la producción y tamizado de numerosos anticuerpos anti-ANG-2 (así como fusiones Fc-péptidos) entre los que se incluye el anticuerpo denominado '536', el cual, tal y como se aprecia en la Tabla 8 de D2 (página 45) presenta el valor IC_{50} más bajo para la unión de ANG-2 con el receptor de tirosina quinasa Tie-2 (el IC_{50} para el anticuerpo 536 de D2 es por lo menos tres veces más bajo que el IC_{50} para los demás anticuerpos revelados en dicha anterioridad, toda vez

¹ $0,04169 \mu\text{g/mL} = 0,00004169 \text{ g/L} = (0,00004169/150000) = 2,8 \times 10^{-10} \text{ mol/L} = 0,28 \text{ nM}$

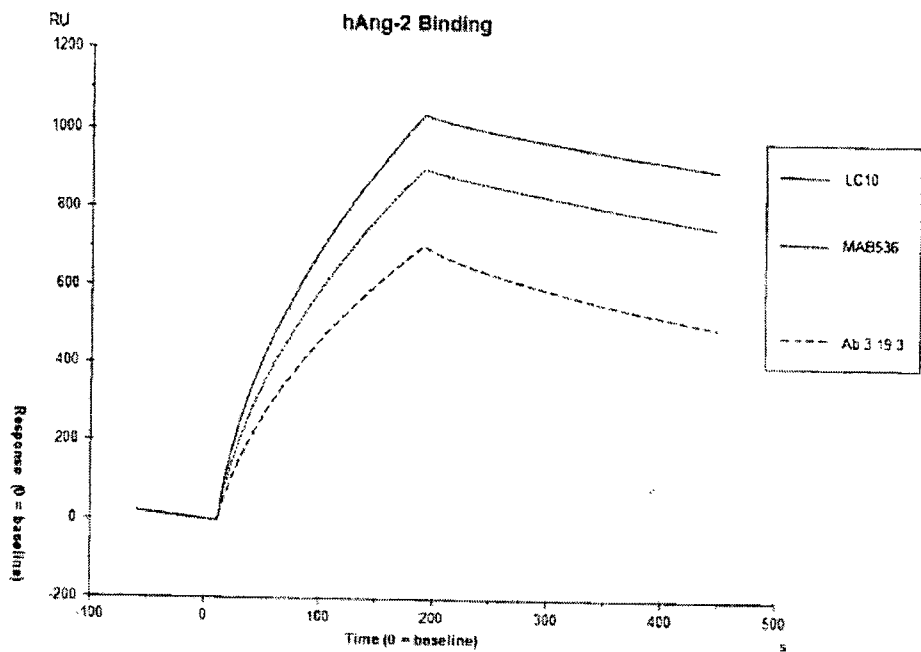
que mientras el anticuerpo Ab 536 muestra un IC_{50} de 0,08 nM para hANG-2 y de 0,05 nM para mANG-2, el anticuerpo con actividad biológica más cercana, esto es el Ab 565, muestra valores IC_{50} de 0,26 nM y 0,26 nM, respectivamente).

Ahora, si bien la actividad biológica de los anticuerpos divulgados en el documento D2 como inhibidores de crecimiento tumoral fue ejemplificada con tres anticuerpos diferentes al anticuerpo 536 (específicamente fueron empleados los anticuerpos 533, 537 y 544 de acuerdo con lo señalado en la página 46, líneas 335 y 336, del documento D2), a partir de lo divulgado por los mismos autores del documento D2 en el artículo científico: *Oliner J. et al.; Cancer Cell. 6 (2004) 507-16* (correspondiente al anexo IV del presente memorial) es absolutamente claro que el anticuerpo AB 536 es el preferido debido a sus bajos valores de IC_{50} para enlazarse a Tie-2, tal como fue previamente analizado. En efecto, en la página 508 (columna derecha, primer párrafo) del artículo científico en cuestión, se afirma lo siguiente:

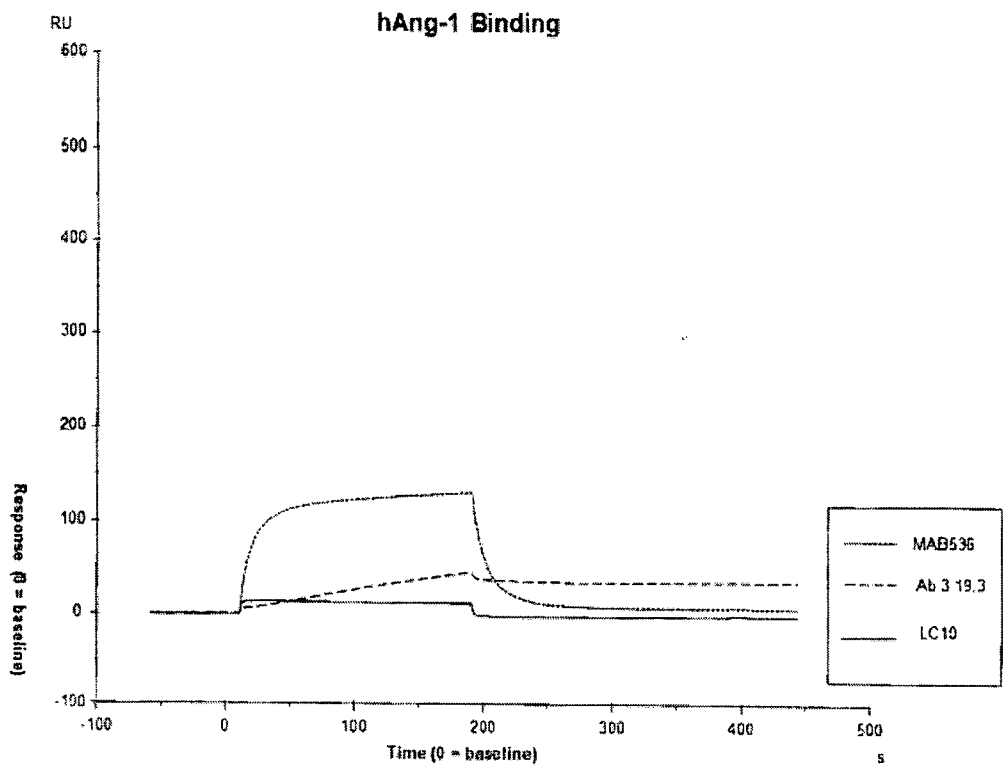
"Proteínas de fusión Fc-péptido y anticuerpos fueron entonces tamizados mediante ELISA de acuerdo con su habilidad para neutralizar la interacción entre Tie2 y angiopoyetinas. Un subconjunto de las proteínas de fusión Fc-péptido fue madurado por afinidad para mejorar su habilidad para neutralizar Ang2. Dos proteínas de fusión Fc-péptido (2xCon4 (C) y L1-7(N)) y un anticuerpo (Ab536) fueron seleccionados para estudios adicionales debido a su capacidad para potencialmente neutralizar la interacción Ang2:Tie2 humana (valores IC_{50} de 23, 54 y 140 pM, respectivamente [Tabla 1A])."

Adicionalmente, quiero llamar la atención sobre los ensayos comparativos entre los anticuerpos <ANG-2> Ang2i_LC10 (LC10) y los anticuerpos Mab536 y Ab3.19.3. En dicho ensayo se compara la unión de los anticuerpos <ANG-2> Ang2i_LC10 (LC10), Mab536 y Ab3.19.3 a los anticuerpos humanos ANG-2 y ANG-1. En este sentido la unión a los anticuerpos humanos ANG-2 y ANG-1 se realizó por medio del ensayo Biacore.

En los resultados de este experimento se observa claramente que el anticuerpo <ANG-2> Ang2i_LC10 (LC10) exhibe unión más fuerte a la angiopoyetina-2 humana (ANG-2) comparada con los Mab536 y Ab3.19.3 revelados en los documentos D1 y D2, tal como se expone en la gráfica a continuación:



Por otro lado, el anticuerpo <ANG-2> Ang2i_LC10 (LC10) no demostró unión a ANG-1, mientras que los anticuerpos Mab536 y Ab3.19.3 si tal y como se muestra en la gráfica a continuación:



Visto lo anterior, es claro que el anticuerpo de la solicitud de patente en cuestión difiere claramente de los anticuerpos revelados por el estado del arte y que los resultados comparativos mencionados son totalmente válidos para demostrar la sorprendente ventaja técnica lograda con el anticuerpo reclamado en la presente invención frente a las enseñanzas de los documentos D1 y D2, toda vez que allí se

compara la especificidad del anticuerpo por la unión a la angiopoyetina-2 humana (ANG-2) y no a la angiopoyetina 1 (ANG-1), lograda con el anticuerpo Ang2i_LC10 de la presente invención frente a la unión exhibida por los anticuerpos Mab536 y Ab3.19.3 conocidos en el estado del arte.

Sumado a lo anterior, la Figura 8 de la memoria descriptiva demuestra la fijación sobre la ANG-1 en donde el anticuerpo Ang2i_LC10 de la presente solicitud de patente no se une a la ANG-1, como se muestra a continuación:

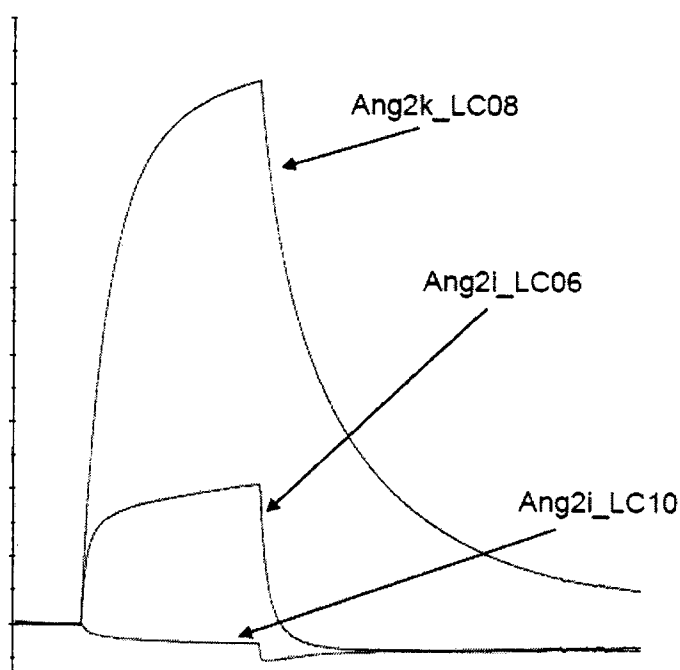


Figura 8 de la solicitud de patente en cuestión, en la cual se divulgan los resultados experimentales de fijación sobre la ANG-1 mediante un Biacore

En efecto, de acuerdo con los datos experimentales revelados en dicha Figura 8 de la solicitud de patente en cuestión (donde las condiciones experimentales empleadas y el análisis de los resultados allí obtenidos son revelados en el ejemplo 7 de la invención, y específicamente en las página 80 de la descripción), es claro que el anticuerpo revelado en la nueva reivindicación 1 muestra que se une específicamente a la angiopoyetina-2 humana (ANG-2), con respecto a la actividad mostrada por los anticuerpos preferidos de los documentos D1 y D2 razón por la cual dicho novedoso anticuerpo presenta un inequívoco carácter inventivo.

Por otra parte, El examinador afirma que el documento D2 divulga una región CDR3 de secuencia la SEQ No 184 del documento D2 la cual presenta un porcentaje de identidad del 88% con la secuencia de aminoácidos de la región CDR3 de SEQ ID No 33, contrario a dicha afirmación me permito presentar el alineamiento de dichas secuencias con el fin de comprobar el porcentaje de identidad y similitud entre las mismas:

JOSE LLORED A CAMACHO & CO.

Calle 72 No. 5-83 Piso 5, Bogot , D.C. Tel: 6069700 Fax 6069701

Secuencia SEQ ID No 33 de presente invenci n:

```
<210> 33
<211> 20
<212> PRT
<213> Artificial
<220>
<223> heavy chain CDR3, <ANG-2> Ang2i_LC10
<400> 33
Ser Pro Asn Pro Tyr Tyr Tyr Asp Ser Ser Gly Tyr Tyr Tyr Pro Gly
1          5          10          15
Ala Phe Asp Ile
                20
```

Secuencia SEQ ID No 184 del documento D2:

```
<211> LENGTH: 16
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 184
Asp Arg Gly Ile Ala Ala Arg Ser Ala Tyr Tyr Tyr Gly Met Asp Val
1          5          10          15
```

Alineamiento/comparaci n de secuencias de la SEQ ID No 33 de la regi n CDR3 de la presente invenci n y la SEQ ID no 184 del documento US 2006/018909 (D2):

```
#=====
#
# Aligned_sequences: 2
# 1: SEQ1
# 2: SEQ2
# Matrix: EBLOSUM62
# Gap_penalty: 10.0
# Extend_penalty: 0.5
#
# Length: 25
# Length of sequence 1: 20
# Length of sequence 2: 16
# Identity:      6/25 (24.0%)
# Similarity:    7/25 (28.0%)
# Gaps:          14/25 (56.0%)
# Score: 16.5
#
```


#=====

SEQ1 SPNPFYYY B D-----SSGYYYPGAFDI 20
 | .|.||| ..|:
SEQ2 ----- 1 DRGIAARSAYYY--GMDV 16

En consecuencia, este alineamiento hace evidente que la identidad entre las dos secuencias corresponde a un 24% y su similitud corresponde a un 28%.

Por todo lo anterior, atentamente me permito manifestar ante ese Despacho que el hecho de que el anticuerpo revelado en la nueva reivindicación 1 de la presente invención muestre una unión específica a (ANG-2) superior, con respecto a los anticuerpos Mad536 y Ab3.19.3 conocidos en el estado de la técnica es una clara prueba del nivel inventivo que la presente invención reviste sobre las enseñanzas de los documentos D1 y D2.

En este sentido, también me permito manifestar ante ese Despacho que el anticuerpo revelado en la presente invención de ninguna manera resulta evidente para la persona medianamente versada en la materia a partir de la combinación de las enseñanzas de los documentos D1 y D2, cumpliendo por lo tanto a cabalidad con las disposiciones de patentabilidad establecidas en el artículo 18 de la Decisión 486 de 2000.

7. **Concesión de la patente en Estados Unidos**

Finalmente y aparte de todo lo arriba mencionado, considero de gran importancia destacar que la solicitud de patente en cuestión fue aceptada por la Oficina de patentes de Estados Unidos y como consecuencia de ello se concedieron la patente US 8,399,626 (Anexo II).

Ante todo, es preciso señalar que con la información mencionada anteriormente de ninguna manera pretendo desconocer la independencia de la que goza la Superintendencia de Industria y Comercio al momento de analizar los méritos de patentabilidad de una solicitud, de acuerdo con las disposiciones del Convenio de París para la protección de la propiedad industrial.

Sin embargo, sí considero de gran importancia resaltar el hecho de que la materia técnica revelada en la solicitud de patente en cuestión ha sido catalogada como novedosa, inventiva e industrialmente aplicable por los Examinadores de la Oficina de Patentes de Estados Unidos, hecho que evidentemente representa un claro indicio de que la solicitud de patente en cuestión cumple a cabalidad con los requisitos de patentabilidad establecidos en la Decisión 486 de 2000.

8. Conclusión

Teniendo en cuenta las modificaciones realizadas en el capítulo reivindicatorio de la solicitud de patente en cuestión y de conformidad con los argumentos presentados para demostrar la claridad, unidad de invención y el nivel inventivo de dicho nuevo capítulo reivindicatorio frente a las enseñanzas de los documentos D1 y D2, resulta completamente claro que se han superado las objeciones expuestas por el señor Examinador en su Concepto Técnico, y por lo tanto, dicha invención es merecedora del privilegio de patente solicitado.

9. Anexo al presente memorial me permito presentar:

- (i) . Copia de las páginas **90** y **91** que corresponde al nuevo capítulo reivindicatorio, el cual está compuesto por **8** reivindicaciones y reemplaza al documento presentado originalmente;
- (ii) Copia de la carátula y el capítulo reivindicatorio de la patente US 8,399,626 concedida el 19 de Marzo de 2013;
- (iii) Copia del artículo científico: " *Buchanan, et al., mAbs 5 (2013) 255 – 262*".
- (iv) Copia del artículo científico: "*Oliner J. et al.; Cancer Cell. 6 (2004) 507-16*".
- (v) Copia de caratula, párrafos 18 y 19 y copia de las secuencias SEQ ID No 3 y 7 del documento WO2009/097325.

10. En vista de lo anterior, ruego a usted proceder a estudiar nuevamente la solicitud a la luz de las modificaciones y los argumentos presentados y conceder el privilegio de patente solicitado.

Señor Superintendente,



ALICIA LLORED A RICAURTE

T. P. 53.215

Reivindicaciones

1. Un anticuerpo o fragmento de anticuerpo que se une específicamente a la angiopoyetina-2 humana (ANG-2), caracterizado porque:

a) El dominio variable de cadena pesada comprende una región CDR3 de la SEQ ID NO: 33, una región CDR2 de la SEQ ID NO: 34 y una región CDR1 de la SEQ ID NO: 35; y,

b) El dominio variable de cadena ligera comprende una región CDR3 de la SEQ ID NO: 36, una región CDR2 de la SEQ ID NO: 37 y una región CDR1 de la SEQ ID NO: 38.

2. El anticuerpo o fragmento de anticuerpo según la reivindicación 1, caracterizado porque comprende:

a) el dominio variable de cadena pesada de la SEQ ID NO: 39; y,

b) el dominio variable de cadena ligera de la SEQ ID NO: 40.

3. El anticuerpo o fragmento de anticuerpo según cualquiera de las reivindicaciones de 1 o 2, caracterizado porque dicho anticuerpo no se une a la angiopoyetina 1 (ANG-1) humana.

4. El anticuerpo o fragmento de anticuerpo según las reivindicaciones 1 a 3, donde dicho anticuerpo es del subgrupo IgG4 humano o es del subgrupo IgG1 humano.

5. Una composición farmacéutica caracterizada porque contiene un anticuerpo o un fragmento de anticuerpo de acuerdo con las reivindicaciones 1 a 3.

6. Un ácido nucleico que codifica la secuencia de aminoácido de un dominio variable de cadena pesada y un dominio variable de cadena ligera según un anticuerpo que se une específicamente a la angiopoyetina-2 (ANG-2) humana, en donde:

- a) el dominio variable de cadena pesada contiene una región CDR3 de la SEQ ID NO: 33, una región CDR2 de la SEQ ID NO: 34, y una región CDR1 de la SEQ ID NO: 35, y
- b) el dominio variable de cadena ligera contiene una región CDR3 de la SEQ ID NO: 36, una región CDR2 de la SEQ ID NO: 37, y una región CDR1 de la SEQ ID NO: 38.

7. Un vector de expresión caracterizado porque contiene un ácido nucleico según la reivindicación 6 para la expresión de un anticuerpo que se une específicamente a la angiopoyetina-2 (ANG-2) humana en un cultivo de célula hospedante procariota o eucariota.

8. Cultivo de Célula hospedante que contiene un vector según la reivindicación 7.

Engineering a therapeutic IgG molecule to address cysteinylolation, aggregation and enhance thermal stability and expression

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¹MedImmune; Cambridge UK; ²MedImmune; Gaithersburg, MD USA; ³AstraZeneca; Waltham, MA USA

Keywords: antibody, aggregation, expression, stability, cysteinylolation

Abbreviations: Ang2, angiopoietin 2; mAb, monoclonal antibody; EPO, erythropoietin; FDA, Food and Drug Administration; QbD, quality by design; V_H, variable heavy; V_L, variable light

Antibodies can undergo a variety of covalent and non-covalent degradation reactions that have adverse effects on efficacy, safety, manufacture and storage. We had identified an antibody to Angiopoietin 2 (Ang2 mAb) that neutralizes Ang2 binding to its receptor in vitro and inhibits tumor growth in vivo. Despite favorable pharmacological activity, the Ang2 mAb preparations were heterogeneous, aggregated rapidly and were poorly expressed. Here, we report the engineering of the antibody variable and constant domains to generate an antibody with reduced propensity to aggregate, enhanced homogeneity, 11°C elevated T_m, 26-fold improved level of expression and retained activity. The engineered molecule, MEDI-3617, is now compatible with the large scale material supply required for clinical trials and is currently being evaluated in Phase 1 in cancer patients. This is the first report to describe the stability engineering of a therapeutic antibody addressing non canonical cysteine residues and the design strategy reported here is generally applicable to other therapeutic antibodies and proteins.

Introduction

Recombinant monoclonal antibodies (mAbs) represent an established and growing class of therapeutics, with more than 20 mAbs approved for the treatment and prevention of disease. It is becoming increasingly apparent, however, that not all candidate mAbs emerging from the drug discovery process are suitable for commercial development, when considering their expression levels, stability and product homogeneity. In particular, the phenomenon of protein aggregation is a common issue that compromises the manufacture, storage, administration, biological activity and safety of biological drugs, including mAbs. In extreme cases, the consequences of biological drug aggregation can be severe. For example, aggregation of the anemia drug erythropoietin (EPO) was one of the factors implicated in EPO-derived immunogenicity that caused pure red cell aplasia, and subsequently fatalities, in patients.¹ Therapeutic antibody immunogenicity is rarely as severe as the case of EPO,² but can still result in unfavorable outcomes, such as patients having to withdraw from therapy.³ The aggregation issue is somewhat exacerbated by the recent move, in the interests of patient convenience, toward subcutaneous self-administration of antibody drugs. In this case, the risk of aggregation is increased due to the

high concentration of antibody required to fill a 1 mL syringe with an effective dose.

Aggregation in the manufacturing process can lead to unwanted heterogeneity in biological protein preparations. Pharmaceutical regulatory authorities, such as the United States Food and Drug Administration (FDA), recommend that heterogeneity be closely monitored and characterized to ensure consistent drug activity between manufacturing lots.⁴ When antibodies are variable in their aggregation profile between production lots, costly monitoring and control procedures are necessary during the manufacturing process. There are many factors that can contribute to protein aggregation, including primary sequence, partial unfolding, post-translational modifications, hydrophobicity, charge, pH, temperature, protein concentration and formulation. Because mAbs are large multidomain proteins, the factors that lead to aggregation are complex and are generally not well understood.⁵ It is becoming standard practice in the industry to select lead antibodies based on both biological activity and aggregation profile. Aggregation propensity can be measured in a number of high throughput assays⁶⁻⁸ and predicted via in silico tools.^{9,10} If aggregation is identified, formulation development is routinely used to minimize aggregation following a 'quality by design' (QbD) approach. Nevertheless, there are limits to

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Table 1. Enhanced profile of Cys49Thr mAb relative to Ang2 mAb

	K_D (pM)	IC_{50} (nM)	% Agg/month at 40°C	T_m 1 (°C)	Yield (mg/L)
Ang2 mAb	24.5	0.2	40	65	10
Cys49Thr mAb	37	0.28	0.2	76	260

Affinity (K_D) was measured by KinExA and activity (IC_{50}) was measured in Ang2/Tie 2 competition ELISA. The rate of aggregation (% Agg/month) was calculated from an accelerated stability study with IgG at 10 mg/ml in 10 mM histidine buffer pH 6 at 40°C. The mid-point of thermal unfolding (T_m) was determined by differential scanning calorimetry. Yield was calculated following transient expression in mammalian cells.

the level of improvement that can be achieved by formulation changes alone. Up to 50% of manufactured product is wasted in some cases.¹¹ Improved variable domain engineering strategies are important to address such issues early in the research phase of drug development to ensure the drug can meet the desired clinical performance.

In the current study, we focused on an antibody targeting angiopoietin 2 (Ang2), a soluble ligand for the Tie2 receptor and an import regulator of pathological angiogenesis and inflammation. The correlation between Ang2 expression in tumors with regions of high angiogenic activity and poor prognosis in many tumor types makes Ang2 an ideal drug target. We previously generated a human anti-Ang2 antibody that neutralizes Ang2 binding to the Tie2 receptor in vitro and inhibits angiogenesis and tumor growth in vivo¹² and now is in clinical trials.¹³ Antibody development was hampered, however, by poor expression and aggregation caused in part by a non-canonical, unpaired Cys residue in the antibody variable domain. Surprisingly, this antibody emerged from a B cell hybridoma screening strategy that should in theory include an intrinsic selection for well-expressed, non-aggregating antibodies.

Antibody stability engineering strategies reported in the literature have focused on improving our general understanding of the residues linked to stability^{9,14-17} or using directed evolutionary strategies to identify aggregation resistant frameworks.^{18,19} Here, we started with an antibody with significant expression and aggregation liabilities and used a rational design approach to engineer the variable domain to reduce aggregation and improve expression. This is the first report of stability engineering addressing non-canonical Cys residues in an antibody and the strategy reported here is applicable to other proteins with unpaired Cys residues to enhance stability and therapeutic use.

Results

Characterization of Ang2 mAb. The variable region genes from an anti-Ang2 hybridoma were cloned into a full-length human IgG2 vector and expressed in mammalian cells. Initial data from the expression and purification highlighted that the yield was significantly lower than expected (Table 1), but the mAb was greater than 99% monomer by SEC-HPLC (data not shown). Sequence analysis of the variable domains and comparison to human germline highlighted a non-canonical Cys at V_L kappa position 49 and an additional non-germline residue at V_H position 37 (Fig. 1A). The presence of non-canonical Cys in antibody germline genes is relatively rare (Fig. 1B). The deconvoluted mass spectra of the N-deglycosylated IgG2 demonstrated heterogeneity of the

antibody (Fig. 2A, upper trace). The theoretical mass of the mAb from the primary sequence is 145,460 Da. Unpaired peptide thiol groups can be modified by the addition of Cys (cysteinylation) or glutathione (glutathiolation) resulting in mass increases of 119 Da and 305 Da respectively. The mass changes from cysteinylation of both unpaired Cys and the combination of cysteinylation and glutathiolation can explain some of the additional peaks in spectra. In addition the degree of Cys modification was variable between antibody expression batches (Fig. S1).

The T_m was calculated by DSC and an accelerated stability study performed to assess the effect of the free Cys on conformational stability and aggregation propensity (Table 1). In brief, the mAb was incubated at 10 mg/ml for two weeks and the proportion of monomer, aggregates and break down products were monitored and used to calculate the rate of aggregation. Ang2 mAb had a very significant rate of aggregation and a relatively low T_m 1 of 65°C (Fig. 2B).

Identification of improved variants enhancing expression and stability with no detrimental effect on immunogenicity and potency. The non-canonical Cys residue was the most likely cause of the aggregation. Structural modeling indicated that it was likely surface-exposed and available for modification (Fig. 1C). The strategy used to engineer the mAb is outlined in Figure 1D. Nineteen variants of Ang2 mAb were screened for activity, mammalian expression and in silico immunogenicity. From these screens, two variants Cys49Asn and Cys49Thr were identified that had improved expression, retained bioactivity and reduced predicted immunogenicity (Fig. 3). To confirm that these variants had improved stability, a miniature accelerated stability study was performed at 1 mg/ml. The two variants retained greater than 90% monomer after 4 weeks, in contrast to the Ang2 mAb which lost 25% monomer (Fig. 4A). The V_H was reverted to germline with the change Gly37Val which had no effect on potency (data not shown). At small scale the variants had improved expression relative to the Ang2 mAb. To further investigate the effect on mAb yield at larger scale, a 5 L transient expression and purification was performed. Both variants had improved expression relative to the Ang2 mAb but, surprisingly, expression of Cys49Thr was more than 10-fold greater than Ang2 mAb and Cys49Asn (Fig. 4B).

Characterization of mAb2. The variant Cys49Thr was characterized relative to the Ang2 mAb in more detail. As expected from previous results, it had significantly improved expression relative the Ang2 mAb and retained affinity and potency (Table 1). The deconvoluted mass spectra of the N-deglycosylated IgG2 demonstrated that the heterogeneity of the antibody was significantly reduced (Fig. 2A). To assess the conformational stability

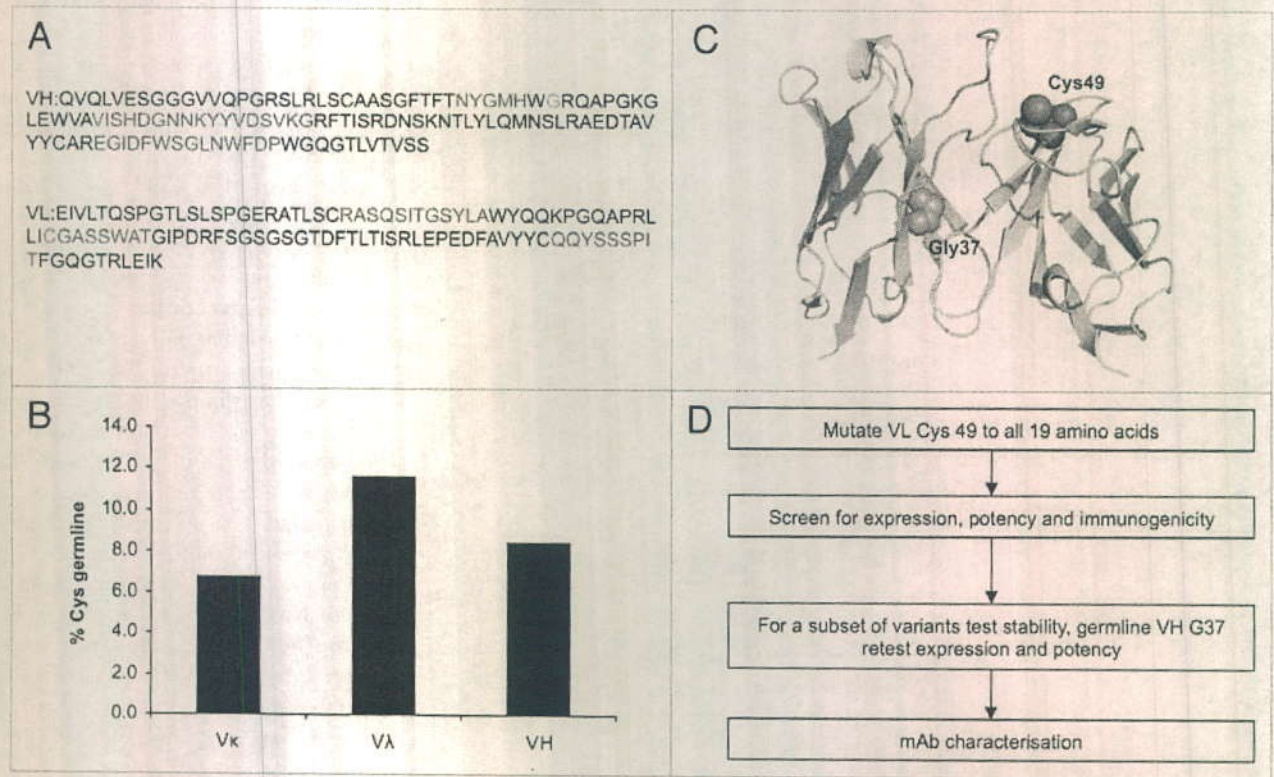


Figure 1. V gene sequence and model of Ang2 mAb. (A) V_H and V_L amino acid sequence with Kabat defined CDRs in blue, non germline framework residue V_H G37 in orange and V_L C49 in red. (B) Frequency of non-canonical Cys residues in human germline genes. (C) Model of Ang2 mAb variable domains highlighting position of V_H G37 (orange) and V_L C49 (red). V_H is in green and V_L in blue. (D) Overview of the strategy used to engineer Ang2 mAb.

and aggregation propensity of the engineered mAb, the T_m was calculated by DSC and an accelerated stability study performed. In brief, the mAb was incubated at 10 mg/ml for two weeks and the proportion of monomer, aggregates and break down products were monitored and used to calculate the rate of aggregation. The T_m was elevated by 11°C (Fig. 2B) and the rate of aggregation significantly reduced (Table 1). Finally, the variant Cys49Thr was converted from human IgG2 to a human IgG1 to remove the isomerisation liability. The IgG1 was characterized as the IgG2 and had an identical profile (data not shown).

Discussion

Therapeutic proteins, including mAbs, are inherently heterogeneous and prone to degradation. The causes of heterogeneity include, but are not limited to, chemical modifications such as deamidation, oxidation and alternative disulfide connectivity. These chemical changes can lead to structural changes that impair the physical stability of the therapeutic protein. Unpaired Cys residues are very reactive and can potentially trigger aggregation through intramolecular scrambling or intermolecular disulfide formation.²⁰⁻²² The Ang2 mAb non-canonical Cys is the cause for the mAb rapid aggregation as demonstrated by the accelerated stability study in Figure 4A. The molecular mechanism of aggregation was not investigated in detail; however, free sulfhydryl groups from unpaired canonical Cys residues are

reported to result in decreased thermal stability.²³ In the case of unpaired non-canonical Cys residues, there are three reports of such mAbs in the literature, MAB007 Cys in V_H CDR3 position 104;²⁴ OKT3 Cys in V_H CDR3 position 100a²⁵ and palivizumab Cys in V_L CDR1 position 25.²⁶ For these three antibodies, the unpaired Cys surprisingly had little effect on stability and aggregation. Palivizumab is a stable molecule,²⁷ OKT3 reported < 1% aggregate after six months at 40°C²⁸ and MAB007 had < 3% aggregate following storage for one month at 45°C.²⁹ The only detrimental effect of the unpaired Cys was the cysteinylated-induced heterogeneity in MAB007.²⁴ Another interesting parallel is the generation of site-specific antibody-drug conjugates based on the introduction of non-canonical Cys residues. Here, the additional Cys residues were modified with Cys or glutathione introducing heterogeneity, as observed for Ang2 mAb, which rendered the Cys inactive as a functional group for conjugation of the payload.^{30,31} The degree of Cys modification of Ang2 mAb was variable, dependent upon cell culture and process conditions. The affect of the Cys on stability is not reported; the affect of Cys on expression was variable and difficult to predict.^{32,33} A structural model of Ang2 mAb was generated and used as the basis for aggregation prediction using computational tools from Accelrys Software Inc. The Ang2 mAb was predicted to have a significant aggregation patch in V_H CDR3 (Fig. S2). The mutation Cys49Thr reduced the predicted aggregation propensity score, while the V_H germline change Gly37Val made no

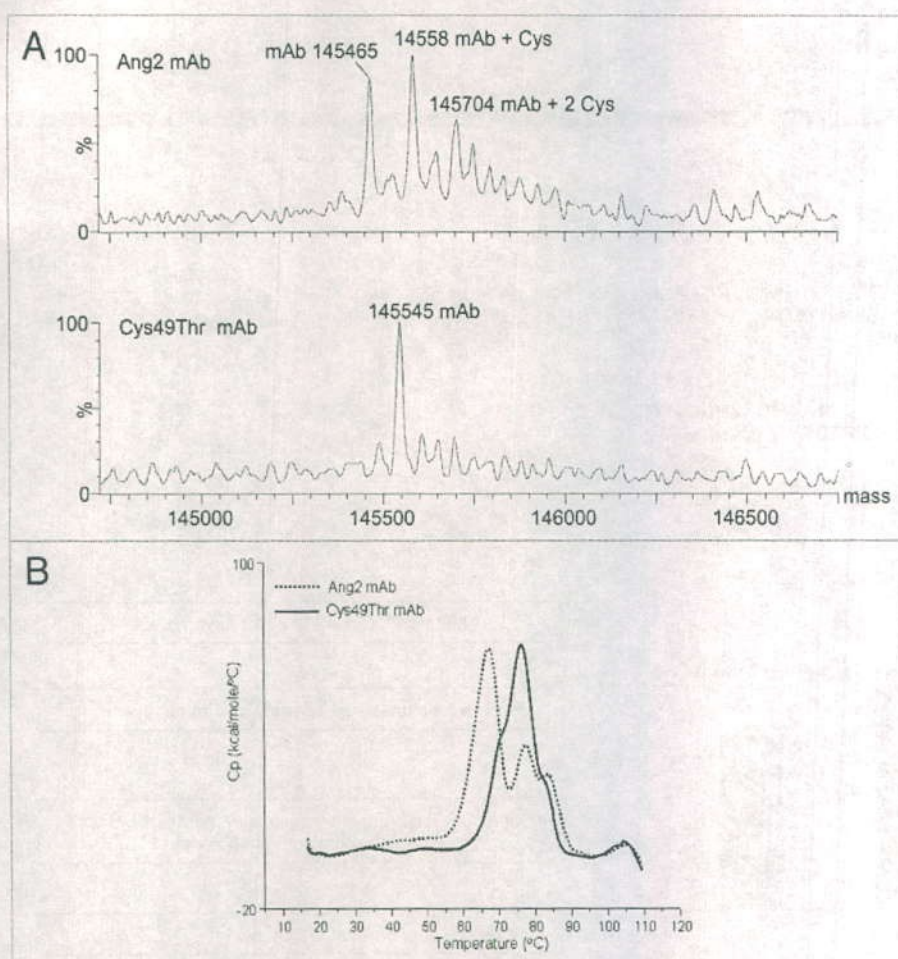


Figure 2. (A) Deconvoluted mass spectra of N-deglycosylated antibodies Cys49Thr mAb and Ang2 mAb. (B) Temperature induced unfolding of the Ang2 mAb and Cys49Thr mAb measured by DSC demonstrating the increased T_m .

impact. The model also suggests that Cys49 is partially exposed and available for modification during expression, purification and storage. V_L Cys 49 in Ang2 mAb may lead to folding problems during expression and trigger aggregation via two related pathways, (1) intramolecular disulfide scrambling and intermolecular disulfide formation and (2) generation of an aggregation prone patch driving self-association.

Protein aggregation causes adverse effects, including reduced efficacy and immunogenicity in patients.^{1,34,35} Two strategies to address aggregation are optimizing the protein sequence through protein engineering or modifying the buffer environment through formulation.³⁶ When introducing amino acid changes into a sequence to reduce aggregation, it is important to maintain activity and not introduce additional T_H cell epitopes that could increase the immunogenicity of the therapeutic molecule. In the case of antibodies, one approach to limit immunogenicity is to mutate residues to the human germline.³⁷ To address the aggregation of Ang2 mAb, a rational rather than evolutionary approach was adopted because V_L 49 Cys was the suspected residue triggering aggregation. Non-canonical Cys are typically rare in the

human germline; however, there are examples of human germline genes encoding unpaired Cys residues, which will be present in platforms that use natural gene sources.³⁸ The typical germline residue at position V_L 49 is Tyr. The mutation Cys40Tyr did improve expression, but significantly reduced the potency of the mAb. The very large Tyr residue is likely to alter the conformation of the proximal V_H CDR3 loop, highlighted in the model of Ang2 mAb. The other large, positively charged or aromatic amino acids also had a detrimental impact on potency, with the exception of Phe. Gly, Ser and Ala are amino acids typically used to replace Cys.²² Their impact on potency was within 2.5-fold, but only Ser and Ala improved expression. Ser and Ala are very small amino acids and, as expected, were potentially acceptable replacements; however, Ala increased the predicted immunogenicity while other variants, such as Asn, Thr, Asp and Glu, demonstrated lower predicted immunogenicity and improved potency relative to Ser.

Variants Asp and Glu had similar expression and potency to Ang2 mAb. Interestingly, the introduction of negatively charged residues, particularly Asp around V_L CDR2, including position V_L 49, has been proposed as a general strategy to reduce the aggregation propensity of antibody scFv.¹⁷ To test whether this observation of scFv translated to full length IgG, we evaluated the variant Cys49Asp in the accelerated stability study. As predicted, Cys49Asp did have a reduced aggregation propensity relative to Ang2 mAb (Fig. S3). The molecular mechanism for this is unclear but this does confirm a preference for small negatively charged amino acids potentially influencing the localized environment, unlike supercharged proteins. This is the first report confirming that small charge changes around V_L CDR2 translates from scFv to improved stability in full-length IgG molecules.

Variant Cys49Asp retained activity, reduced aggregation propensity but did not improve mAb expression. Only two variants Cys49Asn and Cys49Thr improved activity, expression and did not have a detrimental impact on predicted immunogenicity. Asn and Thr are not typically considered as replacements of Cys. Asn and Thr, however, are of a similar size to Cys and, in the context of antibodies, Thr has the potential advantage of neutral hydrophathy that is a hallmark of position V_L 49,³⁹ whereas Asn would introduce a potential Asn Gly deamidation site. Why

Cys49Thr resulted in significantly higher expression than Cys49Asn is not obvious and attempts to predict in silico which mutations would be stabilizing were not successful. In silico tools to predict physicochemical behavior are generally improving but cannot yet replace empirical evidence when attempting to predict how mutations will affect large complex molecules such as IgG. As a final step to ensure Cys49Thr met the desired clinical performance, it was switched from an IgG2 to an IgG1. IgG2 have been shown to aggregate more than IgG1 in manufacture and are prone to disulfide rearrangement in vivo which can reduce activity.^{40,41}

The systematic strategy reported here could be used to rationally engineer other antibody and protein therapeutics with unpaired Cys residues. Previous reports on addressing Cys based stability issues have assessed only a few variants, e.g., studies on IL-2 and G-CSF.^{42,43} The benefit of assessing all potential amino acid changes at a particular site with all the relevant attributes enables an optimal solution to be described by the molecule and the environment. In summary, this is the first report to describe the removal of a significant development liability from a therapeutic mAb candidate, enabling clinical development of the anti-Ang2 antibody. Given the description of other similar liabilities in both mAbs and therapeutic proteins, we believe the approach used here will be applicable to the development of future biopharmaceuticals.

Materials and Methods

Generation of IgG variants, expression and purification. Site-directed mutagenesis was performed using the QuikChange system (Stratagene) according to the manufacturer's instructions. Oligonucleotides were designed to mutate Ang2 mAb V_L position Cys 49 to all other 19 amino acids and variable heavy (V_H) chain Gly 37 to Val. The V_H domain was cloned into a vector containing the human heavy-chain constant domains and regulatory elements to express whole IgG heavy chains in mammalian cells. Similarly, the variable light (V_L) domain was cloned into a vector for the expression of the human light-chain (κ) constant domains and into regulatory elements to express whole IgG light chains in mammalian cells. To obtain IgG proteins, we transfected the heavy-chain and light-chain IgG-expressing vectors into HEK293/EBNA or CHO mammalian cells using standard methods. IgGs were expressed and secreted into the medium. Harvests were pooled and filtered prior to purification. Individual IgGs were purified using Protein A chromatography. Culture supernatants are loaded onto an appropriately sized column of Ceramic Protein A (BioSeptra) and washed with 50 mM

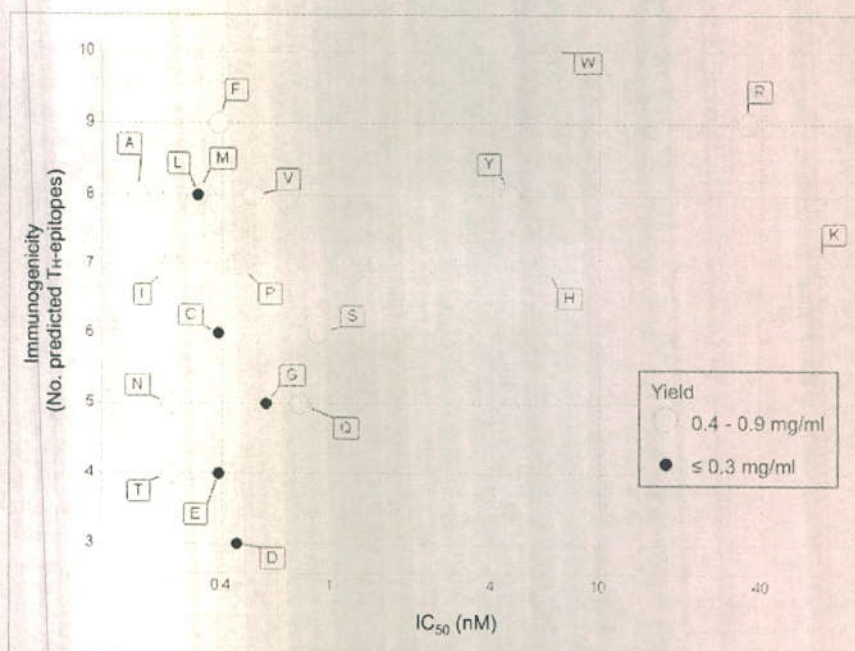


Figure 3. Screening of the Ang2 mAb V_L C49 variants as IgG for activity, predicted immunogenicity and yield. Activity was measured in Ang2/Tie 2 competition ELISA. Immunogenicity was assessed using AlgoNomics Epibase[®] and ranked for the number of predicted T_H-epitopes. IgG yield was determined following transient expression.

Tris-HCl (pH 8.0) and 250 mM NaCl. Bound IgG was eluted from the column using 0.1 M sodium citrate (pH 3) and neutralized by the addition of Tris-HCl (pH 9). The eluted material was buffer exchanged into phosphate-buffered saline (PBS) using Nap10 columns (Amersham). The concentration of IgG was determined at A₂₈₀ using an extinction coefficient based on the amino acid sequence of IgG.

Ang2-Tie2 competition binding ELISA. Maxisorp plates (Nunc) were coated with 4 μ g/ml human Tie2-Fc (R&D Systems), blocked with 0.5% BSA/0.1% Tween-20/PBS buffer and then washed with 0.05% Tween-20/PBS. Anti-Ang2 ranging from 0.4 to 6.6 pM in PBS was added to wells followed by 200 ng/ml biotinylated huAng2 (R&D Systems). Plates were washed, incubated with 1:5000 Streptavidin HRP (Pierce) and developed with TMB substrate (3, 3', 5, 5'-tetramethylbenzidine) solution (KPL). Reaction was stopped and read at OD 450 nm using a Molecular Devices VMAX plate reader (Sunnyvale).

Affinity measurement. The equilibrium binding constant (K_D) for the interaction of IgG with human Ang2 protein was measured using a KinExA platform (Sapidyne Instruments). Briefly, Ang2 protein was coated onto PMMA beads at a concentration of 5 μ g/mL Ang2 using a protocol supplied by the instrument's manufacturer. Afterwards, the beads were washed and blocked in PBS buffer, pH 7.4, containing 10 mg/mL BSA until used. Separately, batch volumes of IgG were prepared at 40 pM and 1 nM in HBS-P buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 0.005% P20) + 0.01% BSA, then aliquoted into two separate sets of 13 tubes each. Human Ang2 protein was

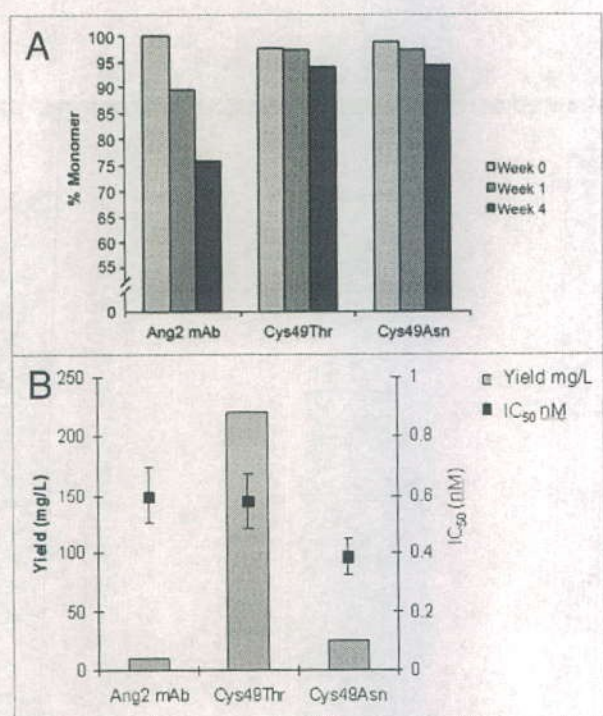


Figure 4. (A) Accelerated stability assessment of variants Cys49Asn, Cys49Thr relative to Ang2 mAb. The IgG samples were incubated at a concentration of 1 mg/ml at 40°C in PBS for 4 weeks and percentage monomer IgG was measured using SEC-HPLC. (B) Activity and yield of the variants. Heavy chain germline change Gly37Val was introduced into Cys49Asn, Cys49Thr and Ang2 mAb IgG. Activity was measured in Ang2/Tie 2 competition ELISA and the yield was assessed following transient expression.

added to one tube from each series and then serially diluted across 11 of the remaining tubes, leaving one sample tube as an antibody-only control. The final Ang2 concentrations for the 40 pM and 1 nM IgG experiments ranged from 313 fM–16 nM and 980 fM–50 nM, respectively. These mixtures were then equilibrated at room temperature for 1–2 d. The Ang2-coated PMMA bead slurries were diluted to ~30 mLs with HBS-N instrument buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl) in a bead vial and affixed to the KinExA instrument. A user-defined timing program was then used to sequentially transfer the Ang2/PMMA beads to a capillary flow cell in the instrument, inject individual sample mixtures (plus or minus Ang2 protein) over the Ang2-coated beads. Following this, unbound sample solution was washed from the beads, and then a species-specific, Cy5-labeled antibody detection reagent, prepared at either 0.0625 µg/mL (1 nM IgG experiment) or 1 µg/mL (40 pM IgG experiment) in HBS-P buffer + 0.01% BSA beads were again washed to remove excess label, then the amount of fluorescence that remained associated with the beads was measured and the signal converted to percent free IgG. Between samples, the bead pack was flushed from the flow cell then replenished with more Ang2-coated beads in preparation for the next sample. An isotherm was then generated, which plotted the amount of free IgG detected at each concentration of

Ang2. The resulting two binding curves were then evaluated in a dual curve analysis using the instrument's evaluation software, from which the K_D was derived.

Mass spectrometry. To profile the IgG, high resolution mass measurement is performed using a liquid chromatography mass spectrometry (LC-MS) method. The LC-MS system is consisted of a Waters Q-TOF (quadrupole and orthogonal acceleration time-of-flight) Premier™ mass spectrometer in conjunction with a Waters ACQUITY UPLC™ (ultra performance liquid Chromatography). To confirm the polypeptide structure, the samples are N-deglycosylated with PNGase F (Calbiochem) after adjusting the pH to 8.0 using 100 mM Tris buffer. Reversed-phased chromatography separation is performed on an Agilent Poroshell C3 75 mm × 2.1 mm column using mobile phase A of 0.01% TFA and 0.1% formic acid in water and mobile phase B of 0.01% TFA and 0.1% formic acid in acetonitrile. Sample is eluted using a fast linear gradient 5–80% mobile phase B over 3 min at a flow rate of 0.12 mL/min. Mass spectrometric data are collected at an m/z range of 2,000 to 4,500. The accurate mass of an antibody is obtained through the deconvolution of the mass spectrometric data using the MaxEnt I (Waters) software package.

Accelerated stability assessment. Purified antibody was tested for stability in an accelerated stability assay. Protein samples were stored at one mg/ml in PBS at 40°C for two weeks. At time zero and week two samples were analyzed by size exclusion chromatography (SEC) using an Agilent 1100 series HPLC system equipped with a TSKG3000SWXL column (Tosoh Bioscience). The column was equilibrated with 0.1 M sodium phosphate, 0.1 M sodium sulfate, 0.05% sodium azide, pH6.8. Samples were injected onto the column and eluted at a flow rate of 1 ml/min with the equilibration buffer. Protein elution was monitored at A_{280} . For quantification purposes, all species eluted before and after the main peak were integrated, and represent aggregates and breakdown products respectively. To confirm the improved stability in a more relevant system antibody was stored at 10 mg/ml in 10 mM histidine pH 6.0 at 40°C for two weeks and the proportion of monomer, aggregates and breakdown products were measured as above. This was used to calculate the rate of aggregation as % aggregation/month after two weeks.

Thermal stability. To calculate the antibody T_m , differential scanning calorimetry (DSC) was performed using a VP capillary DSC system (MicroCal). The test samples were prepared in the buffer alone and in various excipient(s) of interest at a final protein concentration of one mg/mL. Change in the heat capacity (C_p) was measured as the samples were heated from 20°C to 90°C at a rate of 60°C/h. For each sample, a relevant buffer blank containing the same buffer and excipient concentration was used as a reference. Normalized C_p data were corrected for buffer baseline. Data analysis was performed using OriginLab® software (OriginLab Corporation). Raw data from the DSC run were fit using Origin scientific plotting software, which uses the Levenberg–Marquardt nonlinear least square method.

Immunogenicity prediction. The antibody V domains were scanned for the presence of putative T_H -epitopes using Epibase®.⁴⁴ In brief, the platform analyzes the HLA binding specificities of all possible 10 mer peptides derived from the target sequences.

Profiling was done at the allotype level of 20 BRB1, 7 DRB3/4/5, 14 DQ and 7 DP HLA receptors. Peptide corresponding to self peptides were treated separately as 'germline-filtered' peptides.

Disclosure of Potential Conflicts of Interest

All authors are current or former employees of MedImmune.

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Supplemental Materials

Supplemental materials may be found here:

www.landesbioscience.com/journals/mabs/article/23392

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Suppression of angiogenesis and tumor growth by selective inhibition of angiopoietin-2

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Summary

Angiopoietin-2 (Ang2) exhibits broad expression in the remodeling vasculature of human tumors but very limited expression in normal tissues, making it an attractive candidate target for antiangiogenic cancer therapy. To investigate the functional consequences of blocking Ang2 activity, we generated antibodies and peptide-Fc fusion proteins that potently and selectively neutralize the interaction between Ang2 and its receptor, Tie2. Systemic treatment of tumor-bearing mice with these Ang2-blocking agents resulted in tumor stasis, followed by elimination of all measurable tumor in a subset of animals. These effects were accompanied by reduced endothelial cell proliferation, consistent with an antiangiogenic therapeutic mechanism. Anti-Ang2 therapy also prevented VEGF-stimulated neovascularization in a rat corneal model of angiogenesis. These results imply that specific Ang2 inhibition may represent an effective antiangiogenic strategy for treating patients with solid tumors.

Introduction

Conventional cancer therapies often fail to control disease and are toxic by design. To avoid these drawbacks, pharmaceutical efforts are increasingly focusing on therapies that target molecular abnormalities specific to tumorigenesis. While some success has been achieved with such targeted drugs (e.g., STI571, Rituximab, Trastuzumab, Gefitinib, and Erbitux), none of the commercially approved targeted therapies is capable of treating broad populations of cancer patients (Rothenberg et al., 2003). Moreover, while the targeted therapies are generally less toxic than conventional cancer therapies, clinical safety issues have been noted for these drugs. It has been proposed that antiangiogenic cancer therapy might be more broadly effective and safer than either targeted or traditional therapies aimed at cancer cells (Folkman, 1998). Moreover, because antiangiogenic therapy is designed to target blood vessels perfusing tumors, rather than tumor cells themselves, traditional impediments to successful

cancer therapy, such as drug resistance and inadequate drug delivery, might be averted. Promising results have been observed in clinical trials employing such agents (Hurwitz et al., 2003; Kabbinar et al., 2003; Raymond et al., 2002; Yang et al., 2002), particularly with drugs targeting the VEGF-VEGFR pathway, such as Bevacizumab (a humanized VEGF-A binding antibody that was recently approved for the treatment of colon cancer) and SU11248 (a small molecule that inhibits the activity of several protein kinases, including KDR, a VEGF receptor). Nonetheless, clinical efficacy has been mixed for drugs targeting the VEGFs or their receptors (Anonymous, 2002; Miller et al., 2002; Zangari et al., 2004), and significant toxicities have been observed, including fatigue, headaches, hypertension, clotting abnormalities, and life-threatening hemorrhages (DeVore et al., 2000; Kabbinar et al., 2003; Miller et al., 2002).

While the VEGFs and their receptors have been among the most extensively targeted molecules in the angiogenesis field, preclinical efforts targeting the more recently discovered angio-

SIGNIFICANCE

It has been proposed that therapies targeting the tumor vasculature may be more broadly efficacious and less toxic than conventional cancer treatments. Rational design of such therapies involves the identification and characterization of candidate drug targets whose functions are largely restricted to the process of angiogenesis. Ang2, a secreted factor whose expression is upregulated at sites of angiogenesis, holds promise as one such candidate. To evaluate the *in vivo* effects of Ang2 neutralization, we have developed Ang2-selective inhibitors. Systemic anti-Ang2 therapy inhibited tumor growth and angiogenesis in preclinical pharmacology models, and it was well tolerated. Whether Ang2-selective inhibitors will mediate similar effects in humans awaits the evaluation of these therapies in clinical trials.

poietin-Tie2 pathway are now gaining strength (Qian et al., 2002). Both protein families involve ligand-receptor interactions, and both include members whose functions are largely restricted postnatally to endothelial cells (and some hematopoietic stem cell lineages [Iwama et al., 1993]) by virtue of the selective expression of their respective receptors on those cell types (Gale and Yancopoulos, 1999; Mustonen and Alitalo, 1995). Tie2 is a receptor tyrosine kinase with four known ligands, angiopoietin-1 (Ang1) through angiopoietin-4 (Ang4), the best studied being Ang1 and Ang2 (Gale and Yancopoulos, 1999). Whereas Ang1 clearly stimulates phosphorylation of Tie2, Ang2 interaction with Tie2 has been shown to both antagonize and agonize Tie2 receptor phosphorylation, under varying conditions (Davis et al., 1996; Kim et al., 2000; Maisonpierre et al., 1997; Mochizuki et al., 2002; Teichert-Kuliszewska et al., 2001). The phenotypes of mouse Tie2 and Ang1 knockouts are similar and suggest that Ang1-stimulated Tie2 phosphorylation mediates both remodeling and stabilization of developing vessels in utero through maintenance of endothelial cell: support cell adhesion (Dumont et al., 1994; Sato et al., 1995; Suri et al., 1996). The role of Ang1 in vessel stabilization is thought to be conserved in the adult, where the gene is expressed widely and constitutively (Hanahan, 1997; Zagzag et al., 1999). In contrast, normal postnatal Ang2 expression is highest at sites of vascular remodeling (ovary, placenta, and uterus), where it has been proposed to block Ang1 function, thereby inducing a state of vascular plasticity conducive to angiogenesis (Hanahan, 1997; Holash et al., 1999; Maisonpierre et al., 1997). Vessel-selective Ang2 induction has also been demonstrated in disease states associated with angiogenesis. These pathological conditions include cancer, macular degeneration, rheumatoid arthritis, osteoarthritis, and psoriasis (Bunone et al., 1999; Etoh et al., 2001; Fearon et al., 2003; Hangai et al., 2001a; Holash et al., 1999; Kuroda et al., 2001; Otani et al., 1999; Shahrara et al., 2002; Stratmann et al., 1998; Tanaka et al., 1999; Yoshida et al., 1999; Yuan et al., 2000; Zagzag et al., 1999).

Elevated Ang2 expression at sites of normal and pathological postnatal angiogenesis circumstantially implies a proangiogenic role for Ang2 (Maisonpierre et al., 1997). However, experiments designed to functionally confirm this conclusion have yielded ambiguous and contradictory results. For example, ectopic expression of Ang2 in cancer cells has been shown to promote tumor growth and vascularization in xenograft and chorioallantoic membrane angiogenesis models (Ahmad et al., 2001; Etoh et al., 2001; Tanaka et al., 1999). In contrast, Ang2 overexpression has also been reported to inhibit both local tumor growth and metastasis and thereby prolong the survival of mice injected with these tumors (Yu and Stamenkovic, 2001). Experiments using transgenic mice ectopically expressing VEGF, Ang1, and Ang2 in cardiac muscle have demonstrated that Ang2 stimulates VEGF-mediated angiogenesis, whereas Ang1 suppresses it (Visconti et al., 2002). Further supporting the hypothesis that Ang1 and Ang2 play mutually antagonistic roles in developmental vascular remodeling, Ang2 transgenic mice (in which Ang2 expression is driven by the Tie2 promoter) and Ang1 knockout mice share a similar phenotype, consisting of defective vascular patterning, vessel discontinuity, and detachment of the endocardium from the myocardium (Maisonpierre et al., 1997). But since this phenotype implies defects in both vascular remodeling and stabilization, two seemingly contradictory processes, it remains unclear whether either Ang1

or Ang2 are playing pro- and/or antiangiogenic roles in this context. Bolstering the hypothesis that Ang2 is proangiogenic, Ang2 knockout mice display early postnatal vascular remodeling defects in the eye (Gale et al., 2002; Hackett et al., 2002), as well as developmental lymphatic defects; curiously, the latter deficiency, but not the former, can be rescued by replacing the Ang2 gene with the Ang1 gene. Blocking angiopoietin function pharmacologically in a postnatal setting by sequestration with a soluble Tie2 receptor has been shown to have an inhibitory effect on tumorigenesis and neovascularization (Das et al., 2003; Hangai et al., 2001b; Lin et al., 1997, 1998; Siemeister et al., 1999). However, given that all four angiopoietins bind to Tie2, it is not clear whether this effect was mediated by inhibition of Ang1, Ang2, Ang3, Ang4, or a combination thereof. To enable a more definitive evaluation of postnatal Ang2 function, we have generated agents capable of selectively neutralizing the activity of this factor. Here, we show that systemic administration of these Ang2-selective inhibitors is sufficient to suppress both tumor angiogenesis and corneal angiogenesis, thus supporting the notion that Ang2 plays a proangiogenic role postnatally.

Results

Identification and characterization of Ang2-selective inhibitors

Two types of Ang2-selective inhibitors, peptide-Fc fusion proteins and antibodies, were generated as reagents to interrogate angiopoietin function (Supplemental Experimental Procedures at <http://www.cancer.org/cgi/content/full/6/4/507/DC1/>). Phage display peptide and Fab libraries were panned against human Ang2, and the resulting Ang2 binding clones were converted into peptide-Fc fusion proteins (by expressing the active peptides in *E. coli* as fusions to the Fc portion of human IgG1) or fully human antibodies (by expressing the active Fabs in CHO cells on a complete IgG1 framework). Peptide-Fc fusion proteins and antibodies were then screened by ELISA for their ability to neutralize the interaction between Tie2 and angiopoietins. A subset of the peptide-Fc fusion proteins were affinity-matured to enhance their ability to neutralize Ang2. Two peptide-Fc fusion proteins (2xCon4 (C) and L1-7 (N)) and one antibody (Ab536) were chosen for further study, based on their ability to potentially neutralize the human Ang2:human Tie2 interaction (IC_{50} values of 23, 54, and 140 pM, respectively [Table 1A]). All three reagents exhibited similar IC_{50} values against Ang2 from other species (Table 1A), enabling their evaluation in preclinical pharmacology models. Furthermore, the three inhibitors exhibited between 30-fold and >4500-fold selectivity for Ang2 over other angiopoietin family members (Table 1A). Human IgG1 Fc alone exhibited no angiopoietin-neutralizing activity in this ELISA. None of these four agents had activity in a VEGF-VEGFR neutralization assay (Table 1A), whereas Bevacizumab inhibited this interaction with an IC_{50} of 2 nM (data not shown).

To determine practical dosing frequencies and routes of administration for Ang2-blocking agents, pharmacokinetic profiles were generated in rodents for 2xCon4 (C), L1-7 (N), and Ab536 (Table 1B and Supplemental Experimental Procedures). The resulting pharmacokinetic parameters supported the feasibility of daily to weekly subcutaneous dosing.

Ang2 antagonism inhibits tumor growth

To evaluate the effect of Ang2 inactivation on tumor growth, nude mice bearing subcutaneous A431 human epidermoid tu-

Table 1. Peptide-Fc fusion proteins and antibodies as competitive inhibitors of the angiopoietin:Tie2 interaction

A:	Agent	hAng2 IC ₅₀ (nM)	mAng2 IC ₅₀ (nM)	rAng2 IC ₅₀ (nM)	hAng1 IC ₅₀ (nM)	mAng1 IC ₅₀ (nM)	hAng4 IC ₅₀ (nM)	mAng3 IC ₅₀ (nM)	hVEGF IC ₅₀ (nM)
	2xCon4 (C)	0.023	0.021	0.049	0.9	0.64	>100	>100	>100
	L1-7 (N)	0.054	0.071	0.160	>100	>100	>100	>100	>100
	Ab536	0.140	0.061	0.270	>100	>100	>100	>100	>100
	Fc	>100	>100	>100	>100	>100	>100	>100	>100
B:		Mouse		Rat					
	Agent	t _{1/2} (hrs)	Dose-normalized AUC _{0-∞} (nM x hr/ mg/kg)	t _{1/2} (hrs)	Dose-normalized AUC _{0-∞} (nM x hr/ mg/kg)				
	2xCon4 (C) ^a	97	15097	85	8733				
	L1-7 (N) ^a	56	6967	47	4637				
	Ab536 ^b	83	3367	102	12702				

A: Peptide-Fc fusion proteins, antibody, and human IgG1 Fc were evaluated by ELISA for their ability to neutralize angiopoietin:Tie2 interactions. Candidate inhibitory agents were titrated from 100 nM to 0.4 pM in the presence of 1 nM Tie2 and subsequently added to a panel of angiopoietins. The degree of angiopoietin:Tie2 neutralization (IC₅₀) was determined by comparison against a Tie2 standard curve (the binding activity of serially diluted Tie2 in the absence of competitor). As an additional control for specificity, Ang2-inhibitory agents were also tested in an analogous fashion for their ability to neutralize the hVEGF:KDR interaction (last column of table). Bevacizumab (a neutralizing anti-hVEGF antibody) displayed a 2.1 nM IC₅₀ in this assay (data not shown). **B:** Pharmacokinetic parameters of Ang2 inhibitors in mice and rats. Terminal half-lives (t_{1/2}) and areas under the serum concentration-time curves from time 0 to infinity (AUC_{0-∞}) are shown. AUC_{0-∞} values were normalized to a 1 mg/kg dose. ^at_{1/2} and AUC_{0-∞} were calculated from the composite mean serum concentration-time data, n = 2-3/time point. ^bt_{1/2} and AUC_{0-∞} were calculated from the mean serum concentration-time data of 6 mice or 3 rats.

mor xenografts were treated with a range of Ab536 dose levels (thrice weekly) beginning 3 days after tumor challenge (Figure 1A). A431 xenografts, like other tumor models, display vessel-selective Ang2 expression (Figure 1C and Supplemental Experimental Procedures). Ab536 significantly inhibited tumor growth relative to control IgG when dosed at 2 and 6 mg/kg, but not at lower dose levels (Figure 1A). A longer-term study employing Ab536 at 2 mg/kg (the optimal biological dose) showed more dramatic inhibition of tumor growth (Figure 1B). Ab536, as well as the other Ang2 inhibitors, failed to suppress the growth of cultured A431 cells (Figure 1D), consistent with the hypothesis

that the effects of Ab536 on tumor growth in vivo were conferred indirectly through an antiangiogenic mechanism, rather than through a direct effect on tumor cells. We next sought to determine whether the tumor growth inhibition observed in the A431 model could be reproduced in other tumor models. Administration of 2xCon4 (C), L1-7 (N), or Ab536 to mice bearing subcutaneous Colo205 human colorectal tumor xenografts resulted in tumor growth inhibition, indicating that the observed antitumor effects were not cancer type specific or reagent specific (Figure 2A). Vascular Ang2 expression was confirmed by in situ hybridization (Figure 2B and Supple-

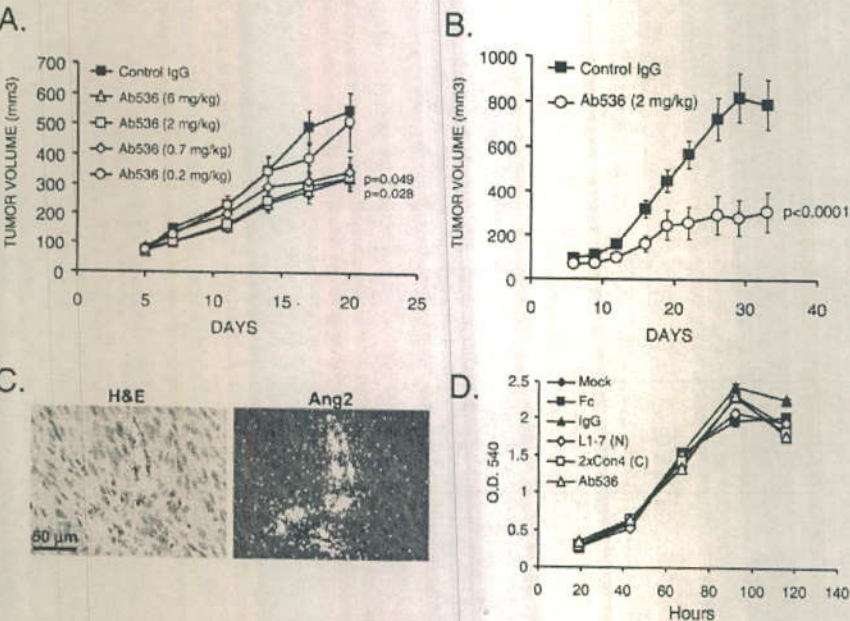


Figure 1. Effect of anti-Ang2 agents on the growth of A431 tumor xenografts and cultured A431 tumor cells. **A and B:** Inhibition of A431 tumor xenograft growth with a systemically administered Ang2-neutralizing agent. Treatment was initiated 3 days postinjection of tumor cells. Ab536 was dosed thrice weekly. **C:** Paired brightfield and darkfield images of Ang2 in situ hybridization, demonstrating Ang2 mRNA expression in endothelial cells lining a blood vessel within the mass of an A431 tumor xenograft from an untreated animal. The horizontal bar indicates scale. **D:** In vitro growth of A431 cells in the presence of Ang2 inhibitors. Cells were seeded in 96-well plates and treated with the indicated concentrations of human Fc, human IgG, L1-7 (N), 2xcon4 (C), and Ab536. Cells were fixed and assayed at 24, 48, 72, 96, and 120 hr of treatment.

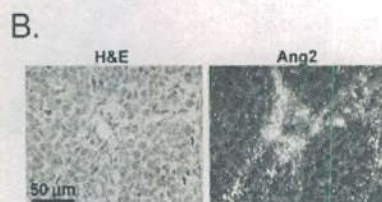
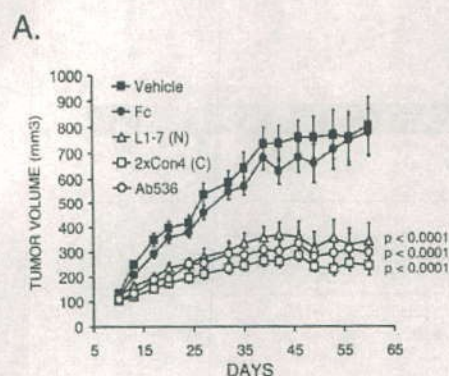


Figure 2. Effect of anti-Ang2 agents on the growth of Colo205 tumor xenografts

A: Inhibition of Colo205 tumor xenograft growth with systemically administered Ang2-neutralizing agents. Treatment was initiated 3 days postinjection of tumor cells.

B: Paired brightfield and darkfield images of Ang2 in situ hybridization, demonstrating Ang2 mRNA expression in endothelial cells lining a blood vessel within the mass of a Colo205 tumor xenograft from an untreated animal. The horizontal bar indicates scale.

mental Experimental Procedures). Ang2 inhibition was shown to mediate similar tumor growth inhibition in the HT29 xenograft model (Supplemental Figure S1).

Two additional Colo205 tumor xenograft experiments were performed to evaluate peptide-Fc fusion protein dose-response relationships. Twice weekly administration of 0.6 or 15 mg/kg 2xCon4 (C) yielded equivalent levels of tumor growth inhibition (Figure 3A), whereas dosing of 0.12 or 0.024 mg/kg 2xCon4 (C) on the same schedule resulted in a progressive reduction in effect relative to the 0.6 mg/kg dose (Figure 3B). The same optimal biological dose of 0.6 mg/kg twice weekly was observed with L1-7 (N) (data not shown).

In the tumor xenograft studies discussed thus far, dosing began 3 days after tumor cells were injected. To better replicate conditions of established disease, Colo205 tumors were allowed to grow for 28 days before 2xCon4 (C) dosing was initiated (Figure 4A). A separation in growth rates between the vehicle and treated groups could be observed within 3 days of treatment initiation, and the gross tumor volume of the treatment group remained static over the 21 days of therapy, indicating that anti-Ang2 therapy could inhibit the growth of established tumors. To examine drug effects at the histological level, cohorts of animals in this study were sacrificed weekly for tumor evaluation. A progressive reduction in viable tumor fraction was observed in the treatment group relative to the vehicle group (Figures 4B–4D and Supplemental Experimental Procedures), indicating that volume-based tumor measurements, which fail to distinguish viable tumor from necrotic tissue, underestimated peptide-Fc fusion protein-mediated antitumor effects. Thus, anti-Ang2 treatment mediated regression of viable tumor.

Even more dramatic regression, apparent even by gross external measurements, was observed when the initiation of therapy was further delayed, and this regression appeared to be reversible following serum clearance of the Ang2-neutralizing agent (Figure 5). In this experiment, 2xCon4 (C) was administered on either a twice weekly schedule (0.6 mg/kg) starting on Day 21 ("narrow-interval dosing") or in individual doses (25 mg/kg) on Days 45 and 116 ("wide-interval dosing"). Dosing on a wide interval allowed 2xCon4 (C) to be cleared from the serum between doses. Rapid tumor regression was observed in the wide-interval group following the Day 45 dose. This regression was sustained until Day 69, at which point tumor regrowth began to occur, suggesting that 2xCon4 (C) exposure had fallen below effective levels. Resumption of dosing on Day 116 resulted again in tumor regression, indicating that tumors had not acquired resistance to anti-Ang2 therapy. While this study showed reversibility of the treatment-mediated antitumor effect, growth inhibition was not observed to be reversible in experiments in which dosing was initiated by experimental Day 28 (data not shown).

To further evaluate the long-term effects of Ang2 inhibition on tumor growth, several groups of animals harboring A431 and Colo205 tumor xenografts were treated continuously with 2xCon4 (C) or Ab536 for at least 10 weeks (Supplemental Table S1 and Supplemental Experimental Procedures). Although various dosing levels and schedules were employed, all were predicted to result in serum trough levels exceeding the exposure required for optimal effect. In 13 of 15 groups, anti-Ang2 therapy eliminated all measurable tumor in a subset of animals. In three of these groups, drug was withdrawn after the complete responses were achieved, and no tumor regrowth was observed

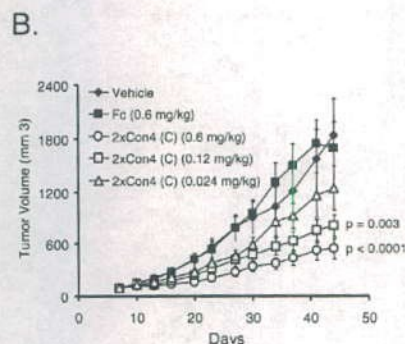
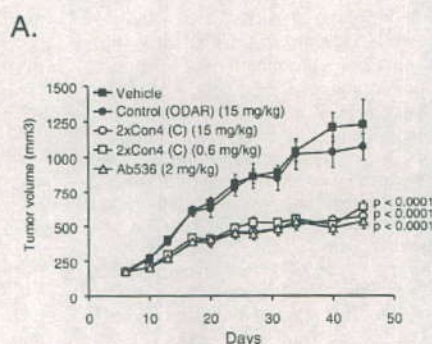


Figure 3. Dose-response effect of systemically administered 2xCon4 (C) in the Colo205 xenograft model

A: 2xCon4 (C) dose levels of 15 and 0.6 mg/kg (twice weekly) had similar antitumor effects to one another and to Ab536 dosed at 2 mg/kg (thrice weekly).

B: Reducing the 2xCon4 (C) doses levels to 0.6, 0.12, and 0.024 mg/kg (twice weekly) resulted in a clear dose-response effect. While maximal effect was observed with 0.6 mg/kg, a partial inhibitory effect was observed with the intermediate dose of 0.12 mg/kg. The low dose of 0.024 mg/kg was ineffective.

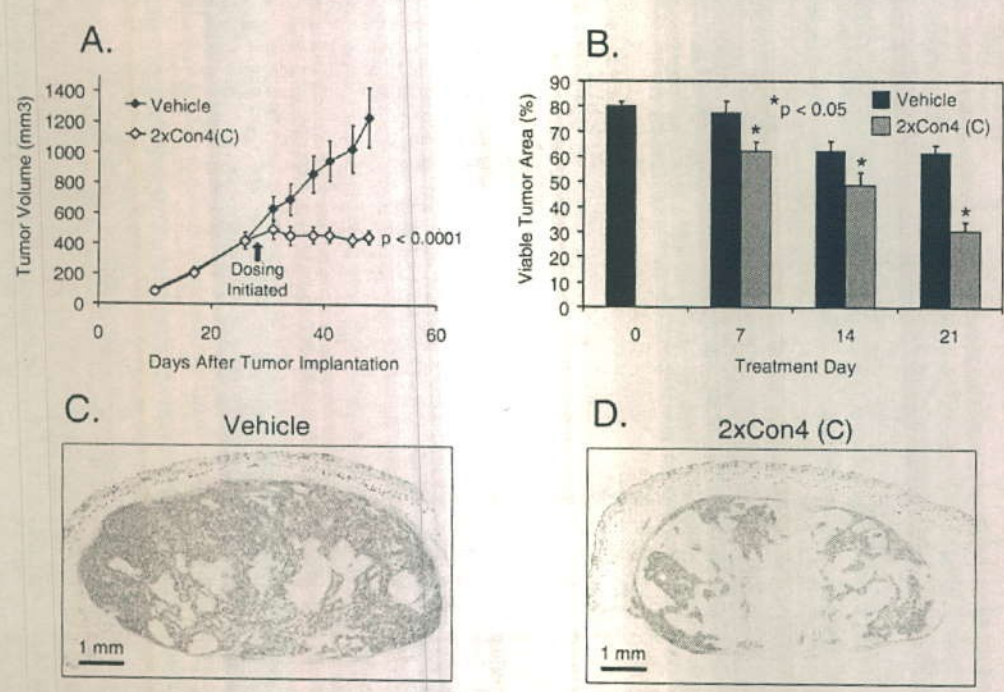


Figure 4. Effect of 2xCon4 (C) treatment on viable tumor fraction in the Colo205 xenograft model
A: Mice were treated twice weekly with 15 mg/kg 2xCon4 (C) starting on day 28 postinjection of Colo205 tumor cells. Tumors were harvested at different time points following treatment initiation for the histological analysis described below. Representative tumor volumes from mice treated with 2xCon4 (C) or vehicle for 21 days are shown.
B: Analysis of tumor viable area fraction comparing 2xCon4 (C)-treated mice and vehicle-treated mice. Eight tumors per group were measured on posttreatment days 0, 7, 14, and 21. A significant reduction in tumor viability was demonstrated following 7 days of 2xCon4 (C) treatment.
C and D: Representative hematoxylin-stained histological sections of bisected tumors following 21 days of treatment with vehicle or 2xCon4 (C), respectively. The horizontal bars indicate scale.

in these animals (average follow-up 15.3 weeks; range 6–27 weeks).

Upon completion of tumor xenograft studies, mice were sacrificed to search for possible adverse effects in major organ

systems attributable to Ang2 neutralization (Supplemental Experimental Procedures). The 2xCon4 (C) protein had no significant adverse effects on xenograft-bearing mice when dosed either at 14 mg/kg twice per week for 11 weeks (monitoring hematology values, serum chemistry values, or histology) or 8 mg/kg daily for 11 weeks (monitoring organ weights and histology). Similarly, L1-7 (N) had no untoward effects on any of the endpoints evaluated (hematology values, serum chemistry, or histology) in xenograft-bearing mice when dosed at 14 mg/kg twice per week for 7 weeks.

Ang2 inhibition suppresses the proliferation of tumor endothelial cells

To begin investigating the mechanism through which Ang2 neutralization mediates tumor growth inhibition, Colo205 xenografts from mice treated with 2xCon4 (C) or vehicle were evaluated at the cellular level. FACS analysis revealed that 2xCon4 (C) treatment resulted in a progressive reduction in endothelial cell proliferation, followed by a reduction in tumor cell proliferation (Figure 6A and Supplemental Experimental Procedures), suggesting that the 2xCon4 (C)-mediated antitumor effects occurred secondary to a drop in tumor perfusion. That is, while 24 hr of 2xCon4 (C) treatment was sufficient to inhibit endothelial cell proliferation, 72 hr of 2xCon4 (C) treatment was required to achieve a reduction in tumor cell proliferation that deviated below the aggregate mean tumor cell proliferation of all vehicle-

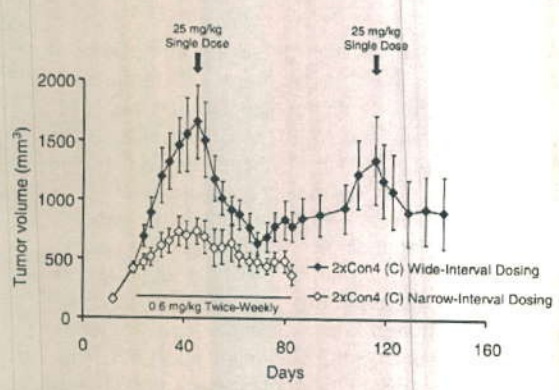


Figure 5. Systemically administered 2xCon4-C induces regression of large established tumors
 One group of mice was treated twice weekly with 15 mg/kg 2xCon4 (C) beginning on Day 21 postinjection of cells ("Narrow-Interval Dosing" group). The other group was treated with vehicle until Day 44 and then with 25 mg/kg doses of 2xCon4 (C) on Days 45 and 116 ("Wide-Interval Dosing" group).

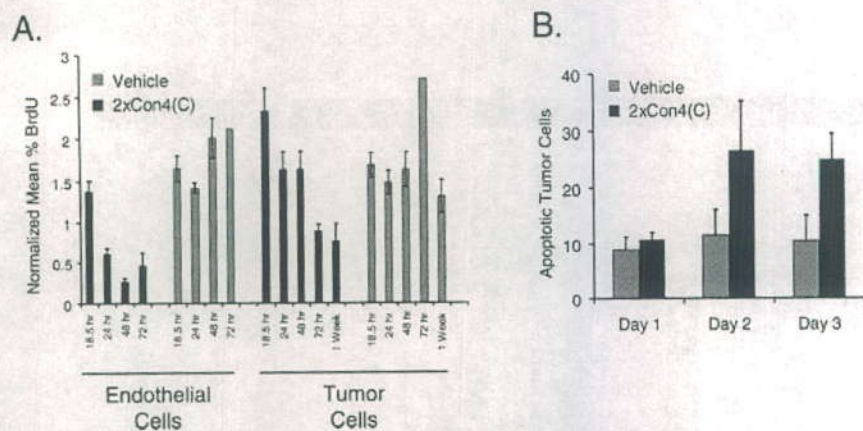


Figure 6. Cell-based mechanism-of-action studies

A: Effect of 2xCon4 (C) on BrdU uptake in tumor cells and endothelial cells derived from Colo205 xenografts. Tumor-bearing mice were treated with a single dose of 15 mg/kg 2xCon4 (C) or vehicle at study day 28. Tumors were collected at different times posttreatment and digested enzymatically, and cells were extracted for FACS analysis. 18 hr prior to each tumor harvest, mice were implanted subcutaneously with osmotic minipumps (Zymed Labs 2001D, 8 μ l/hr, 1 day) containing 3 mg/ml BrdU. Dissociated cells were stained with anti-mouse CD45-FITC and CD31-PE antibodies, followed by fixation and staining with anti-BrdU-alexa647 antibodies. Endothelial cell (CD45⁺/CD31⁺ mouse diploid population) and tumor cell (CD45⁺/CD31⁺/FSC tetraploid population) BrdU uptake was normalized

to total diploid mouse cell BrdU uptake for each tumor sample. The bar graph represents normalized mean BrdU ratios ($n = 4$, except for 72 hr vehicle group [$n = 1$] and 72 hr 2xCon4 group [$n = 2$]).

B: Apoptotic tumor cell counts per 20 $\times$ objective field (135,600 μ m²), comparing 2xCon4 (C)-treated and vehicle-treated mice. Eight tumors per group were evaluated following 1, 2, or 3 days of treatment. This analysis demonstrated a significant increase in tumor cell apoptosis following 2 days of 2xCon4 (C) treatment.

treated groups (tumor cell proliferation in the vehicle-treated groups remained relatively constant over time). Additional analyses by histological methods demonstrated that 2xCon4 (C) induced tumor cell apoptosis in Colo205 xenografts (Figure 6B), an effect that is consistent with the reduced viable tumor fraction observed in 2xCon4 (C)-treated mice (Figures 4B–4D). The temporal and causative relationships between 2xCon4 (C)'s effects on tumor cell proliferation and apoptosis are not yet known.

Direct antiangiogenic effect mediated by Ang2 neutralization

We next sought further support for the hypothesis that anti-Ang2 therapy mediates a direct antineovascular effect *in vivo*. The rat corneal angiogenesis model was chosen for this purpose, as it allows neovascularization to be studied outside the context of tumorigenesis. In this model, a VEGF-soaked nylon disk is placed in the normally avascular cornea at a fixed distance from the surrounding limbal vessels. Neovessels subsequently sprout from the limbal vessels toward the developing VEGF gradient. *In situ* hybridization (ISH) was used to demonstrate that Ang2 was expressed in the VEGF-induced corneal neovasculature (Supplemental Figure S2 and Supplemental Experimental Procedures). Ang2 mRNA levels were shown to be upregulated during VEGF-induced corneal angiogenesis in a punctate pattern similar to that observed in tumor vessels. Ang2 ISH signal was identified only in neovessels sprouting from the limbus, and no signal could be observed in the absence of exogenous VEGF stimulation. The consequences of inactivating Ang2 in this model were then evaluated. Systemic administration of 2xCon4 (C) over a range of 0.125 to 4 mg/kg twice weekly resulted in a dose-dependent inhibition of VEGF-mediated corneal angiogenesis (Figure 7A), with reduction of angiogenesis to negative control levels observed at the 1 mg/kg dose level. L1-7 (N) and Ab536 were also shown to inhibit corneal angiogenesis to background levels (Figure 7B), further demonstrating that the effects of anti-Ang2 therapy were not reagent specific.

Treatment of rats with L1-7 (N) and 2xCon4 (C) at subcutaneous doses as high as 200 mg/kg twice weekly for 2 weeks

was well tolerated and revealed no significant adverse effects (Supplemental Experimental Procedures). A pharmacological effect on epiphyseal growth plate thickness was observed in male 2xCon4 (C)-treated rats dosed at 75 mg/kg (2 of 3 rats) and 200 mg/kg (3 of 3 rats). No growth plate abnormalities were observed in identically treated rats that were subsequently withdrawn from drug for 1 month, suggesting that these effects were reversible. Epiphyseal plate thickening is considered to be a pharmacological consequence of antiangiogenic therapy and has been seen in growing rodents treated with VEGF-neutralizing antibodies (Gerber et al., 1999). This phenomenon would not be expected to occur in adult animals treated with antiangiogenic agents, as endochondral bone formation is completed before adulthood. Also of note, a multifocal infiltration of mononuclear cells was seen on the exterior surface of the splenic capsule in some treated rodents, but not primates (data not shown). The finding had no known functional consequences, and its etiology is under investigation. Together, the accumulated preclinical pharmacology data support the conclusion that Ang2 inactivation suppresses angiogenesis in both neoplastic and nonneoplastic settings, without untoward effects on animal health.

Discussion

Previous work in this field has highlighted the complexity of Ang2 function. *In vitro* and *in vivo* experiments have suggested that Ang2 can either agonize or antagonize (Davis et al., 1996; Kim et al., 2000; Maisonpierre et al., 1997; Mochizuki et al., 2002; Teichert-Kuliszewski et al., 2001) the Tie2 receptor, depending on context. Ang2 has been shown to play opposing roles at the cellular and organismal levels as well, promoting either vascular sprouting or regression, depending on whether VEGF is present (Maisonpierre et al., 1997). The phenotype of the recently published Ang2 knockout mouse is informative in that it convincingly implicates Ang2 as a required factor for early postnatal retinal neovascularization (Gale et al., 2002; Hackett et al., 2002). However, these studies also highlight the context-dependent nature

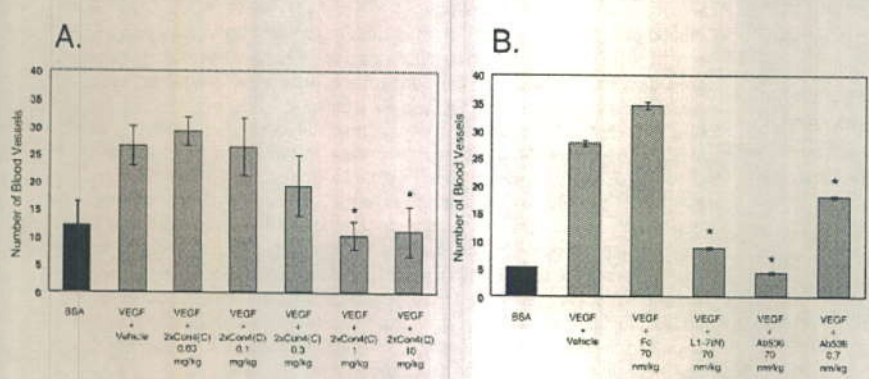


Figure 7. Ang2 antagonists inhibit VEGF-induced corneal angiogenesis

Angiogenesis was induced by implanting VEGF- or BSA-soaked nylon discs into the corneal stroma of female CD rats. Treatment with 2xCon4(C) (A), Fc control or L1-7(N) (B) was initiated 1 week prior to corneal implantation and continued twice per week. Animals receiving Ab536 (B) were injected on the day of surgery and three days later. For all groups, angiogenesis was evaluated 1 week after disc implantation. Values are the group mean $\pm$ standard error of the mean ($n = 8$). * $p < 0.02$.

of Ang2 dependency in angiogenesis; Ang2 was shown in these same experiments to be dispensable for developmental neovascularization and for pathological angiogenesis in the abnormally retained hyaloid vasculature of rare knockout mice surviving to day 25 (Gale et al., 2002). In tumor models, ectopic Ang2 expression has been shown to both stimulate and inhibit tumor growth and tumor angiogenesis, thus adding to the confusion regarding Ang2 function. The only published attempt to selectively neutralize Ang2 in vivo using a pharmacological agent (an RNA aptamer) resulted in incomplete angiogenic inhibition, even in the context of local administration, thus leaving unanswered the question of whether Ang2 inhibition alone is sufficient to fully inhibit angiogenesis (White et al., 2003). The cellular mechanism through which selective Ang2 inhibition mediated angiogenic suppression also remains to be addressed. Panangiopoietin inhibition (with soluble Tie2) has also been shown to mediate suppression of tumor growth and angiogenesis, but none of these effects can be attributed specifically to Ang2 inactivation (Das et al., 2003; Hangai et al., 2001b; Lin et al., 1997, 1998; Siemeister et al., 1999). It therefore remains unclear exactly what molecular, cellular, and phenotypic roles Ang2 plays in postnatal life in normal or pathological vascular processes, and particularly in the setting of cancer.

To begin investigating this question, we generated Ang2-selective peptide-Fc fusion proteins and antibodies, enabling controlled pharmacological inactivation of endogenous Ang2. This approach to Ang2 inhibition circumvents the neonatal lethality observed in Ang2 knockout studies (Gale et al., 2002) and avoids the ambiguous interpretations inherent in studies using panangiopoietin inhibitors (Das et al., 2003; Hangai et al., 2001b; Lin et al., 1997, 1998; Siemeister et al., 1999). Our studies demonstrate that specific inactivation of endogenous Ang2 inhibits tumor angiogenesis in mice and corneal angiogenesis in rats, thus establishing that Ang2 plays a proangiogenic role in these processes. A combination of volume-based tumor measurements and histological analysis indicate that selective Ang2 inhibition mediates regression of viable tumor. Moreover, a subset (~20%) of tumor-bearing mice completely responded to long-term anti-Ang2 therapy, in that no residual tumor could be measured. This result suggested that even established tumor vessels have the capacity to respond to Ang2 inhibition.

No significant adverse effects were observed in rodents following long-term Ang2 inhibition, despite clear Ang2 expression by ISH in several organs of 10-week-old mice (data not shown). This result is reminiscent of the Ang2 knockout mouse

phenotype (Gale et al., 2002), in which no prenatal vascular defects were observed, despite strong embryonic expression of Ang2 in and around large vessels in wild-type mice. Thus, expression of Ang2 does not necessarily confer functional dependence on this factor. Nonetheless, additional studies of longer duration and in the context of other angiogenesis-associated processes (such as wound healing and estrous cycling) would be necessary to fully assess the effects of Ang2-neutralizing agents.

While this study sheds light on Ang2 biology and the therapeutic implications of Ang2 inhibition, it also stimulates additional questions. First, the cellular and molecular consequences of Ang2 neutralization require further investigation. It remains to be determined, for example, how Ang2 neutralization blocks endothelial cell proliferation, if it alters pericyte/cell matrix coverage of capillaries, or how it might alter tumor perfusion. Of note, ectopically administered Ang2 has been shown to promote endothelial cell proliferation and sprouting of new blood vessels in the papillary membrane of the eye, and these effects were associated with increased capillary diameter, basal lamina remodeling, and enhanced endothelial cell migration (Lobov et al., 2002). It is attractive to consider that these same associated characteristics might be oppositely affected by Ang2 inhibition. At the molecular level, it remains unclear how Ang2 neutralization affects Tie2 phosphorylation in individual vessels. Attempts to address this question by immunohistochemistry using anti-phospho-Tie2 antibodies have not been successful, as the reagent antibodies generated for this purpose have not been sufficiently sensitive. Evaluating this question in a physiologically relevant setting is critical to elucidating the true biochemical role of Ang2.

A second question that remains to be addressed is whether selective inhibition of Ang1 would be advantageous or deleterious in terms of efficacy and safety. If, for example, Ang1 and Ang2 were both capable of stimulating tumor angiogenesis, then greater tumor growth inhibition might be achievable by neutralizing both angiopoietins, rather than inhibiting Ang2 alone. Alternatively, if Ang1 and Ang2 function in a mutually antagonistic manner, then selective Ang1 neutralization might actually stimulate tumor growth. From the standpoint of safety, one might predict, a priori, that Ang1 inhibition could globally destabilize normal quiescent blood vessels, as Ang1 appears to play a role in normal vessel maintenance. Systemically administered soluble Tie2 has been reported to be efficacious and safe in tumor models (Lin et al., 1998), but interpretation of

these studies is confounded by the fact that Ang1 and Ang2 inhibition could not be evaluated in isolation. While 2xCon4(C) selectively inhibits Ang2, it too displays some inhibitory activity against Ang1. Despite this Ang1-neutralizing activity, 2xCon4(C) shares a similar response profile with the more Ang2-selective reagents (L1-7(N) and Ab536) evaluated herein, suggesting that (1) Ang2 may be the dominant angiopoietin in promoting postnatal angiogenesis and (2) inhibiting Ang1 may confer no deleterious effects. Nonetheless, definitive evaluation of the consequences of inhibiting Ang1 awaits the generation of high-potency Ang1-selective inhibitors and has implications for pharmaceutical strategies involving targeted inactivation of the angiopoietins or their receptor.

A final question involves determining the clinical value of selectively inhibiting Ang2. Ang2 upregulation has been observed not only in cancer, but also in macular degeneration, rheumatoid arthritis, osteoarthritis, and psoriasis (Bunone et al., 1999; Etoh et al., 2001; Fearon et al., 2003; Hangai et al., 2001a; Holash et al., 1999; Kuroda et al., 2001; Otani et al., 1999; Shahrara et al., 2002; Stratmann et al., 1998; Tanaka et al., 1999; Yoshida et al., 1999; Yuan et al., 2000; Zagzag et al., 1999), suggesting that an effective Ang2 inhibitor might have wide applicability in treating human disease. Whether Ang2-selective inhibitors will confer similar activity in humans to that observed in rodent models awaits the introduction of these agents into clinical trials.

Experimental procedures

Angiopoietin:Tie2 neutralization ELISA

96-well microtiter plates were coated with recombinant angiopoietins in 293T cell conditioned media (DMEM/50 μ g/ml BSA) at 37°C for 1 hr. The conditioned media were used at angiopoietin concentrations that conferred 75% of maximally achievable binding to 1 nM hTie2-Fc (R&D Systems, catalog #313-TI). Plates were washed with PBS/0.1% Tween-20 and then blocked for 2 hr at room temperature with PBS/5% BSA. Peptide-Fc fusion proteins and antibodies titrated from 100 nM to 0.4 pM in a solution of PBS/1% BSA/1 nM Tie2 were added to the angiopoietin-coated plates, which were incubated overnight at room temperature and then washed with PBS/0.1 percent Tween-20. Mouse-derived anti-Tie2 antibody (Pharmingen Inc., catalog #557039) was added to each plate at a final concentration of 1 μ g/ml (1 hr incubation at room temperature), after which plates were washed in PBS/0.1% Tween-20. Goat anti-mouse-IgG-HRP (Pierce, catalog #31432) was added at a dilution of 1:10,000 in PBS/1% BSA (1 hr incubated at room temperature), after which plates were washed several times with PBS/0.1% Tween-20. TMB substrate (Sigma, catalog #T8665) was added, OD 370 nm absorbance was measured, and degree of angiopoietin:Tie2 neutralization was determined by comparison against a Tie2 standard curve.

VEGF/KDR neutralization ELISA

96-well microtiter plates were coated with recombinant human VEGF (0.1 ml per well of 5 μ g/ml hVEGF in PBS/1% BSA) at 37°C for 1 hr. The recombinant VEGF was used at concentrations that conferred 80% of maximally achievable binding to 1 nM human KDR-Fc (R&D Systems, catalog #357-KD/CF). Plates were washed with PBS/0.1% Tween-20 and then blocked for 2 hr at room temperature with PBS/5% BSA. Peptide-Fc fusion proteins and antibodies titrated from 1000 nM to 0.5 pM in a solution of PBS/1% BSA/1 nM KDR-Fc were added to the VEGF-coated plates, which were incubated overnight at room temperature and then washed with PBS/0.1% Tween-20. Mouse-derived anti-KDR antibody (R&D Systems, catalog #MAB3573) was added to each plate at a final concentration of 1 μ g/ml (1 hr incubation at room temperature), after which plates were washed in PBS/0.1% Tween-20. Goat anti-mouse-IgG-HRP (Pierce, catalog #31432) was added at a dilution of 1:5000 in PBS/1% BSA (1 hr incubated at room temperature), after which plates were washed several times with PBS/0.1% Tween-20. TMB substrate (Sigma, catalog #T8665) was added, OD 370 nm absorbance was measured,

and degree of VEGF:KDR neutralization was determined by comparison against a KDR-Fc standard curve.

Pharmacology studies

8- to 10-week-old female CD1 nude mice (Charles Rivers Laboratories) were used in all studies. Mice were injected subcutaneously with 0.2 ml of tumor cell suspension in RPMI, containing 1×10^7 A431 cells (ATCC), 2×10^6 Colo205 cells (ATCC), or 5×10^6 HT29 cells mixed with 2:1 volumes matrigel:cells (BD Bioscience). Subconfluent cells were harvested prior to injection. Treatment with peptide-Fc fusion proteins, antibodies, or vehicle (PBS) was initiated as early as 3 days and as late as 45 days postinjection of cells. Peptide-Fc fusion proteins were administered subcutaneously, and antibodies were administered intraperitoneally at the indicated doses and schedules. Tumor volumes and body weights were recorded at regular intervals. Tumor volume was calculated as length $\times$ width $\times$ height in mm³. Results are expressed in mean $\pm$ standard error. The data were statistically analyzed with factorial ANOVA followed by Scheffe's post hoc analysis for repeated measurements (StatView v5.0.1, SAS Institute). Mice were euthanized with CO₂ asphyxiation, and in some experiments tumors were collected and fixed for histological analysis. All experiments were conducted in accordance with institutional guidelines, which include mandates for sacrificing mice when tumors exceed size thresholds.

In vitro cell growth assay

A431 cells were seeded in 96-well tissue culture plates at 2000 cells per well in 200 μ l of DMEM supplemented with 10% fetal bovine serum (FBS). 16 hr postseeding, the medium was replaced with 100 μ l per well of fresh DMEM/10% FBS containing 6.7 μ M Ang2-neutralizing agents or controls (all in triplicate). Cells were fixed at 24, 48, 72, 96, and 120 hr post treatment by adding 100 μ l 10% trichloroacetic acid (TCA). All plates were stored in 10% TCA at 4°C and assayed together. The 10% TCA was shaken out, and the wells were rinsed 5 times with water. The cells were then stained with 100 μ l of 0.4% sulforhodamine B (Sigma S-9012) in 1% acetic acid (Sigma A-6283) for 10 min at room temperature and then washed 5 times with 1% acetic acid. The plates were then air-dried. The dye was solubilized with 300 μ l of 20 mM unbuffered Tris (pH > 10) for 2 hr on a rotary shaker. Optical density (OD) was then read at 540 nm on a microtiter plate reader.

Tumor cell apoptosis assay

Tumor cell apoptosis was examined at days 1, 2, and 3 for both 2xCon4 (C)-treated and vehicle-treated controls, from the same study as the Viable Tumor Fraction analysis. Sections were stained for cleaved Caspase-3 (Cat#-Asp175, Cell Signaling Technologies) using peroxidase localization with DAB substrate, and a light hematoxylin counterstain. Five widely spaced fields were selected in a blinded fashion from the circumferential region of eight tumor sections per group, avoiding the overlying capsule and areas of frank necrosis. The number of apoptotic tumor cells was counted in each 20 $\times$ objective field of roughly 135,600 μ m².

Corneal angiogenesis model

Corneal angiogenesis was induced in adult female CD rats weighing 250–300 g ($n = 8$ per group) as described (Coxon et al., 2002). Briefly, 0.6 mm diameter circular discs of Nyalflo filter paper (Gelman, Ann Arbor, MI) were incubated in PBS containing either 0.1% bovine serum albumin (BSA) or 0.1% BSA with 10 μ M vascular endothelial growth factor (VEGF) (R&D Systems, Minneapolis, MN) for 1 hr at 4°C. Rats were anesthetized with isoflurane and a single disc was inserted into a pocket in the corneal stroma. The margin of each disc was located $\sim$ 1.8 mm from blood vessels of the lateral limbus.

Treatment with 2xCon4 (C) (0, 0.03, 0.1, 0.3, 1.0, or 10 mg/kg), Fc control (70 nM/kg), L1-7(N) (70 nM/kg), or vehicle (PBS) was initiated 1 week prior to corneal implantation and continued twice per week. Rats treated with Ab536 (Ab536 0.7 or 70 nM/kg) received one injection on the day of surgery and another 3 days later. All treatments were injected subcutaneously.

7 days after surgical implant, the corneas were photographed at 25 $\times$ using a Nikon SV-3 Ophthalmic Slit Lamp (Nikon Ophthalmic) equipped with a Nikon D-1 digital camera back. A reference stage micrometer was photographed for calibration. Numerical data were generated from the digital images using Metamorph image analysis software (v4.5; Universal Imaging,

Downingtown, PA). For each corneal image, the number of vessels intersecting the midpoint between the disc and the limbus was measured. Values represent the group mean $\pm$ standard error of the mean. Statistical significance was assessed by analysis of variance followed by Fisher's exact test.

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(54) **ANTIBODIES AGAINST HUMAN
ANGIOPOIETIN 2**

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(57) **ABSTRACT**

The present invention relates to antibodies against human Angiopoietin 2 (anti-ANG-2 antibodies), methods for their production, pharmaceutical compositions containing said antibodies, and uses thereof.

5 Claims, 13 Drawing Sheets

79

The invention claimed is:
1. An isolated antibody which binds specifically to human
angiopoietin-2(ANG-2), wherein said antibody comprises:
(A) a heavy chain variable domain which comprises a
CDR3 region having the sequence of SEQ ID NO: 33, a 5
CDR2 region having the sequence of SEQ ID NO: 34
and a CDR1 region having the sequence of SEQ ID NO:
35;
(B) a light chain variable domain which comprises a CDR3
region having the sequence of SEQ ID NO: 36, a CDR2 10
region having the sequence of SEQ ID NO: 37 and a
CDR1 region having the sequence of SEQ ID NO: 38.

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2. An antibody according to claim 1, comprising a heavy
chain variable domain having the sequence of SEQ ID NO:
39; and a light chain variable domain having the sequence of
SEQ ID NO: 40.
3. An antibody according to claim 1 wherein said antibody
does not bind to human Angiopoietin 1 (ANG-1).
4. An antibody according to claim 1 wherein said antibody
is of the human IgG4 subclass or the human IgG1 subclass.
5. A pharmaceutical composition comprising an antibody
according to claim 1 or a fragment thereof.

* * * * *

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(54) Title: STABILIZED ANGIOPOIETIN-2 ANTIBODIES AND USES THEREOF

(57) Abstract: Stabilized antibodies directed to Angiopoietin-2 and uses of such antibodies are described. Nucleic acid and amino acid sequences, hybridomas or other cell lines for expressing such antibodies are also provided.

5 [017] Antibodies of the invention have the ability to specifically bind Ang-2 and inhibit tumor angiogenesis and reduce tumor growth. Mechanisms by which this can be achieved can include, but are not limited to, either inhibition of binding of Ang-2 and/or Ang1 to its receptor Tie2, inhibition of Ang-2 and/or Ang-1 induced Tie2 signaling, inhibition of Ang-2 and/or Ang-1 induced Tie2 phosphorylation, or increased clearance of Ang-2 and/or Ang1, 10 therein reducing the effective concentration of Ang-2 and/or Ang-1.

[018] In one embodiment, the antibodies of the invention exhibit enhanced stability as compared to the control Ang-2 specific antibody 3.19.3. In another embodiment, the antibodies of the invention exhibit enhanced production yields as compared to a control Ang-2 specific antibody. In one embodiment, the antibody of the invention is a human Ang-2 15 antibody which comprises a Val substitution at position 37 of the heavy chain as defined by the EU numbering system ((Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, Fifth Edition, NIH Publication 91-3242, Bethesda MD (1991), vols. 1-3.), and a substitution of Asp, Thr, Asn, or Ala at position 49 of the light chain based on the Kabat numbering system.

20 [019] In another embodiment, the antibodies of the invention may comprise a variable light chain amino acid sequence selected from the group consisting of MEDI1 (SEQ ID No.:3), MEDI2 (SEQ ID No.:4), MEDI3 (SEQ ID No.:5), MEDI6 (SEQ ID No.:8) and MEDI4 (SEQ ID No.:6). In another embodiment, the antibodies of the invention may comprise the heavy chain variable amino acid sequence MEDI5 (SEQ ID No.:7). In another embodiment, the 25 antibodies of the invention may comprise a variable light chain amino acid sequence selected from the group consisting of MEDI1 (SEQ ID No.:3), MEDI2 (SEQ ID No.:4), MEDI3 (SEQ ID No.:5), MEDI6 (SEQ ID No.:8) and MEDI4 (SEQ ID No.:6) as well as a heavy chain variable sequence defined as MEDI5 (SEQ ID No.:7).

[020] In another aspect, the invention also provides nucleic acid sequences, vectors and cell 30 lines for expression of the antibodies of the invention.

[021] The invention further provides methods for assaying the level of Angiopoietin-2 (Ang-2) in a patient sample, comprising contacting an anti-Ang-2 antibody with a biological sample from a patient, and detecting the level of binding between said antibody and Ang-2 in said sample. In more specific embodiments, the biological sample is blood.

35 [022] In other embodiments the invention provides compositions, including an antibody or functional fragment thereof, and a pharmaceutically acceptable carrier.

[023] Further embodiments include methods of effectively treating an animal suffering from an angiogenesis-related disease, including selecting an animal in need of treatment for a

5 MEDI1 light chain SEQ ID No:3

EIVLTQSPGTLSSLSPGERATLSCRASQSITGSYLA^WYQQKPGQAPRLLI^TGASSWATGIPDR
FSGSGSGTDFTLTISRLEPEDFAVYYCQQYSSSPITFGQGRLEIKRTVAAPSVFIFPPSDE
10 QLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDYSLSSSTLTLSKAD
YEKHKVYACEVTHQGLSSPVTKSFNRGEC

MEDI2 light chain SEQ ID No:4

EIVLTQSPGTLSSLSPGERATLSCRASQSITGSYLA^WYQQKPGQAPRLLI^NGASSWATGIPDR
15 FSGSGSGTDFTLTISRLEPEDFAVYYCQQYSSSPITFGQGRLEIKRTVAAPSVFIFPPSDE
QLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDYSLSSSTLTLSKAD
YEKHKVYACEVTHQGLSSPVTKSFNRGEC

MEDI3 light chain SEQ ID No:5

20 EIVLTQSPGTLSSLSPGERATLSCRASQSITGSYLA^WYQQKPGQAPRLLI^DGASSWATGIPDR
FSGSGSGTDFTLTISRLEPEDFAVYYCQQYSSSPITFGQGRLEIKRTVAAPSVFIFPPSDE
QLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDYSLSSSTLTLSKAD
25 YEKHKVYACEVTHQGLSSPVTKSFNRGEC

MEDI4 light chain SEQ ID No:6

EIVLTQSPGTLSSLSPGERATLSCRASQSITGSYLA^WYQQKPGQAPRLLI^AGASSWATGIPDR
30 FSGSGSGTDFTLTISRLEPEDFAVYYCQQYSSSPITFGQGRLEIKRTVAAPSVFIFPPSDE
QLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDYSLSSSTLTLSKAD
YEKHKVYACEVTHQGLSSPVTKSFNRGEC

MEDI5 heavy chain SEQ ID No:7

35 QVQLVESGGGVVQPGRSLRLSCAASGFTFTNYGMHW^VIRQAPGKGLEWVAVISHDGNMKYYVD
SVKGRFTISRDNKNTLYLQMNSLRAEDTAVYYCAREGIDFWSGLNWFD^PWGQGT^LLVTVSS

MEDI6 light chain SEQ ID No:8

40 EIVLTQSPGTLSSLSPGERATLSCRASQSITGSYLA^WYQQKPGQAPRLLI^TGASSWATGI^ADR
FSGSGSGTDFTLTISRLEPEDFAVYYCQQYSSSPITFGQGRLEIKRTVAAPSVFIFPPSDE
QLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDYSLSSSTLTLSKAD
45 YEKHKVYACEVTHQGLSSPVTKSFNRGEC

VII. EXAMPLES:

Example 1: Potency of modified Ang-2 antibodies

[0249] In this example, Ang-2 specific antibodies comprising the light and heavy chains of
50 the Ang-2 antibody 3.19.3 have been modified such that amino acid substitutions for cysteine
at position 49 of the light chain were introduced. The potency of the resultant antibodies was
measured in an Ang-2:Tie-2 potency assay. The results are presented in Table 1 below.