

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO	
RAD: 13-135221- -5	FECHA: 2015-05-16 23:15:21
DEP: 2020 DIRECCION DE NUEVAS CREACIONES	EVE: 378 FASE NACIONAL INCLUIDO CAPITULO I
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II	FOLIOS: 28
ACT: 430 REQUERSOLICITA	

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
JUAN PABLO CADENA SARMIENTO

Asunto: Radicación: 13-135221- -5
 Trámite: 11
 Evento: 378
 Actuación: 430
 Oficio: 5265
 Folios: 28

Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I	X	Capítulo II	
No. Solicitud Internacional	PCT/US2011/065895				
Fecha de Presentación Internacional	19/12/2011				

Como resultado del examen de fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Radicado N° 13-135221-00000-0000	04/Junio/2013
Reivindicaciones:	Radicado N° 13-135221-00000-0000	04/Junio/2013
	Radicado N° 13-135221-00004-0000	22/Enero/2014
Oposiciones:	Radicado N° 13-135221-00001-0000	Lafrancol 29/Octubre/2013
Respuesta de la solicitante:	Radicado N° 13-135221-00004-0000	22/Enero/2014
Prioridad:	US61/459,962	20/Diciembre/2010

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque ésta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del capítulo primero del Título 10 de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por la solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada. En el presente caso se estudiarán las reivindicaciones 1 a 47 del capítulo reivindicatorio (Radicado N° 13-135221-00004-0000).

Título de la solicitud: “Anticuerpos anti-mesotelina e inmunoconjugados”.

El problema planteado en esta solicitud consiste en la necesidad de proporcionar compuestos útiles en el tratamiento de desórdenes asociados a la mesotelina.

Para resolver este problema técnico la solicitud en estudio proporciona un anticuerpo que se une a mesotelina seleccionado de (i) un anticuerpo que se une un epítope de SEQ ID N° 43 que comprende E153 y D174.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): C07K 16/30.

4. Excepción a la Patentabilidad (Art 20 D 486 literal (d))

El objeto de las reivindicaciones 39-47 no es patentable de acuerdo con el Art citado. Considerando que el centro de las reivindicaciones corresponde a un método de diagnóstico de enfermedades asociadas a la mesotelina.

5. De la solicitud de patente

Titulo

El título no corresponde con el objeto de la solicitud porque es amplio y no determina la materia que se busca proteger, adicionalmente hace alusión a métodos de tratamiento, por lo cual se sugiere a la solicitante hacer la corrección necesaria.

Reivindicaciones (Art 30 D 486, Art 22 PCT)

La reivindicación 1 no es clara porque caracteriza un anticuerpo de manera incorrecta, se le recuerda a la solicitante que un anticuerpo se define en términos de sus cadenas ligera y pesada utilizando las siglas SEQ ID NO. En el presente caso la solicitante caracteriza el anticuerpo de acuerdo al epítope de unión lo cual es incorrecto, considerando que permitiría un sinnúmero de posibles moléculas que se unan a este y que no se encuentran sustentadas.

La reivindicación 1 no es clara porque caracteriza a un anticuerpo en términos de la constante de afinidad que corresponde a un resultado a alcanzar y no definen el anticuerpo reclamado, puesto que la reivindicación incluiría no sólo la solución propuesta por la solicitante, sino todas las alternativas presentes o futuras que lleguen a ese resultado. Se sugiere reemplazarlo por las características esenciales, estructurales o funcionales, que contribuyen a la obtención de ese efecto.

La reivindicación 2 no es clara porque caracteriza el anticuerpo de manera incorrecta, se le recuerda a la solicitante que un anticuerpo se caracteriza en términos de su cadena ligera y pesada, utilizando las siglas SEQ ID NO: o los 6 CDRs de las cadenas

ligera y pesada, en el presente caso la solicitante únicamente lo caracteriza en términos de los 3 CDRs de la cadena pesada.

La reivindicación 3 no es clara porque caracteriza el anticuerpo de manera incorrecta, se le recuerda a la solicitante que un anticuerpo se caracteriza en términos de su cadena ligera y pesada, utilizando las siglas SEQ ID NO: o los 6 CDRs de las cadenas ligera y pesada, en el presente caso la solicitante únicamente lo caracteriza en términos de los 3 CDRs de la cadena ligera.

La reivindicación 4 no es clara porque define tres anticuerpos que presentan secuencias diferentes de cadena ligera y pesada. Se sugiere colocar cada anticuerpo en una reivindicación independiente.

Las reivindicaciones 5, 6 y 7 no son claras porque definen de manera incorrecta un anticuerpo en términos de su cadena ligera. Se le aclara a la solicitante que un anticuerpo se define en términos de su cadena ligera y pesada.

La reivindicación 8 no es clara porque comprende la misma materia de la reivindicación 4, fundamentando que el anticuerpo que comprende la secuencia de cadena pesada SEQ ID NO: 8 y la cadena ligera SEQ ID NO:4. Contiene los tres CDRs de la cadena pesada SEQ ID NO: 20, 21 y 22 y los tres CDRs de la cadena ligera SED ID NO: 11, 18 y 19 definidos en la reivindicación 4 como se muestra a continuación:

Alineamiento SED ID NO: 8 y CDRs de SEQ ID N°: 20, 21 y 22:

CLUSTAL 2.1 multiple sequence alignment

```
SEQID16Pesada      EVQLVESGGGLVQPGGSLRLSCAASGYFTTTYWMHWVRQAPGKGLEWVG YIRPSTGYTEY 60
CDR1SEQIDN°20      -----GYTFTTYWMH----- 10
                      *****
```

```
SEQID16Pesada      NQKFKDRFTISADTSKNTAYLQMNSLRAEDTAVYYCARSRWLLDYWGQGLTVTS 115
CDR1SEQIDN°20      -----
```

CLUSTAL 2.1 multiple sequence alignment

```
SEQID16Pesada      EVQLVESGGGLVQPGGSLRLSCAASGYFTTTYWMHWVRQAPGKGLEWVG YIRPSTGYTEY 60
CDR1SEQIDN°21      -----GYIRPSTGYTEY 12
                      *****
```

```
SEQID16Pesada      NQKFKDRFTISADTSKNTAYLQMNSLRAEDTAVYYCARSRWLLDYWGQGLTVTS 115
CDR1SEQIDN°21      NQKFKD----- 18
                      *****
```

CLUSTAL 2.1 multiple sequence alignment

```
SEQID16Pesada      EVQLVESGGGLVQPGGSLRLSCAASGYFTTTYWMHWVRQAPGKGLEWVG YIRPSTGYTEY 60
CDR3SEQIDN°22      -----
```

```
SEQID16Pesada      NQKFKDRFTISADTSKNTAYLQMNSLRAEDTAVYYCARSRWLLDYWGQGLTVTS 115
CDR3SEQIDN°22      -----ARSRWLLDY----- 9
                      *****
```

Alineamiento SED ID NO: 4 y CDRs de SEQ ID N°: 17, 18 y 19:

CLUSTAL 2.1 multiple sequence alignment

```
SEQID4Ligera      DIQMTQSPSSLSASVGDRVTITCKSSQSVLYSSNQKNYLAWFQQKPGKAPKLLIYWASTR 60
CDR1SEQIDN°17     -----KSSQSVLYSSNQKNYLA----- 17
                      *****
```

```
SEQID4Ligera      ESGVPSRFSGSGSGTDFTLTISLQPEDFATYFCHQYLSSYTFGQGTKVEIKR 113
CDR1SEQIDN°17     -----
```

CLUSTAL 2.1 multiple sequence alignment

```
SEQID4Ligera      DIQMTQSPSSLSASVGDRVTITCKSSQSVLYSSNQKNYLAWFQQKPGKAPKLLIYWASTR 60
CDR2SEQIDN°18     -----WASTR 5
                      *****
```

```
SEQID4Ligera      ESGVPSRFSGSGSGTDFTLTISLQPEDFATYFCHQYLSSYTFGQGTKVEIKR 113
CDR2SEQIDN°18     ES----- 7
                      **
```

CLUSTAL 2.1 multiple sequence alignment

```
SEQID4Ligera      DIQMTQSPSSLSASVGDRVTITCKSSQSVLYSSNQKNYLAWFQQKPGKAPKLLIYWASTR 60
CDR3SEQIDN°19     -----
```

```
SEQID4Ligera      ESGVPSRFSGSGSGTDFTLTISLQPEDFATYFCHQYLSSYTFGQGTKVEIKR 113
CDR3SEQIDN°19     -----HQYLSSYT----- 8
                      *****
```

Las reivindicaciones 11 y 14, no son claras porque caracteriza un anticuerpo en términos del epítipo de unión lo cual permite un sinnúmero de posibilidades que no se encuentran sustentadas, adicionalmente se le recuerda a la solicitante que un anticuerpo no puede ser definido en términos de su epítipo de unión.

Las reivindicaciones 15, 16 y 17, no son claras porque no definen el anticuerpo de manera correcta en términos de su cadena pesada y ligera, se sugiere eliminar o hacer las aclaraciones necesarias.

Las reivindicaciones 20 y 21 no son claras porque define el anticuerpo en términos del epítipo de unión.

La reivindicación 24 no es clara porque reclama un ácido nucleico de manera incorrecta, en el presente caso la solicitante debe definir la SEQ del ácido nucleico mediante el cual se obtienen los anticuerpos de la solicitud

La reivindicación 27 no se encuentra sustentada en el capítulo descriptivo, considerando que la expresión “agente citotóxico”, corresponde a un grupo de moléculas que tiene actividad citotóxica. En el presente caso no se encuentra sustento en el capítulo descriptivo para todas las moléculas que tienen esta actividad se sugiere eliminar del capítulo reivindicatorio o hacer las aclaraciones necesarias.

La reivindicación 27 no es clara porque el término “L es unión”, es impreciso y no es conocido en el campo de los inmunoconjugados.

Las reivindicaciones 34 a 38 no se encuentran sustentadas en el capítulo descriptivo porque la materia reclamada en estas no se encuentra divulgada en el capítulo descriptivo, considerando que no existe sustento para todos conjugados que comprenden los tres anticuerpos descritos en la reivindicación 8 de la presente solicitud. Adicionalmente no existe sustento para las composiciones que comprenden estos inmunoconjugados se sugiere eliminar del capítulo reivindicatorio.

6. Unidad de invención (Art 25 D 486)

No hay unidad de invención entre las cuatro reivindicaciones independientes ya que el concepto común inventivo, anticuerpos de anti-mesotelina, no cumple con el requisito nivel inventivo de acuerdo con los documentos D1 y D3 (Análisis de patentabilidad para la reivindicación 8).

Por lo tanto, esta invención presenta 4 diferentes conceptos técnicos comunes inventivos así:

- Grupo I: Parcialmente reivindicaciones 1-12. Anticuerpo que comprenden la secuencia de cadena pesada SEQ ID N°8 y la secuencia de cadena ligera SEQ ID N°4. Divulgado en el documento D1, D2 y D3.
- Grupo II: Parcialmente reivindicaciones 1-14. Anticuerpo que comprenden la secuencia de cadena pesada SEQ ID N°16 y la secuencia de cadena ligera SEQ ID N°12. Divulgado en el documento D1, D2 y D3.
- Grupo III: Parcialmente reivindicaciones 1-24. Anticuerpo que comprenden la secuencia producida por el hibridoma 19C3. Divulgado en el documento D1, D2 y D3.
- Grupo IV: Parcialmente reivindicaciones 28-38. Conjugados de fórmula (I) que comprenden un anticuerpo de cadena pesada SEQ ID N°16 y la secuencia de cadena ligera SEQ ID N°4. Divulgados en el documento D4 y D5.
- Grupo V: Parcialmente reivindicaciones 28-38. Conjugados de fórmula (I) que comprenden un anticuerpo de cadena pesada SEQ ID N°16 y la secuencia de cadena ligera SEQ ID N°12. Divulgados en el documento D4 y D5.
- Grupo VI: Parcialmente reivindicaciones 28-38.. Conjugados de fórmula (I) que comprenden un anticuerpo de cadena pesada SEQ ID N°8 y la secuencia de cadena ligera SEQ ID N°4. Divulgados en el documento D4 y D5.

Se le sugiere a la solicitante restringir la materia a patentar en un solo concepto común inventivo especificando el avance tecnológico sobre el estado de la técnica. Esto se logra entre otras formas definiendo las características esenciales de la invención y mostrando las otras características particulares como dependientes, pero sin perjuicio de la concisión de las nuevas reivindicaciones tanto independientes como dependientes.

La solicitante también puede realizar una o más de las divisionales de acuerdo a los grupos de conceptos técnicos inventivos presentados en esta solicitud, esto claro, sin ampliar el objeto inicial de la invención.

7. Análisis de la Oposición

Argumentaciones de Lafrancol

Por su parte la sociedad opositora Lafrancol S.A afirma que esta solicitud no cumple con los requisitos de patentabilidad, novedad, nivel inventivo, aplicación industrial. Considerando que las composiciones y combinaciones farmacéuticas que se refieren a anticuerpos anti-mesotelina e inmunoconjugados así como a métodos de uso de los

mismos, ítuiles en el tratamiento de cáncer en la presente solicitud, no presentan ninguna clase de novedad ni de nivel inventivo, por lo tanto el opositor afirma que no es pertinente conceder un beneficio patentario.

La opositora manifiesta sobre las composiciones farmacéuticas hace mención general de anticuerpos, sin especificar alguno, lo cual da a entender que la presencia del anticuerpo es opcional. Por lo que concluye que no hay certeza ni explicación suficiente sobre la materia a patentar y considera que lo anterior controvierte la las disposiciones comunitarias, en lo referente al requisito de unidad inventiva.

Asimismo el opositor afirma que no puede existir beneficio patentario para reivindicaciones de composiciones y combinaciones farmacéuticas útiles en el tratamiento del cáncer y que comprenden moléculas plenamente identificadas y utilizadas. Y agrega que la solicitud de la referencia pretende proteger mediante derechos patentarios objetos que no son considerados invenciones e indica que la mezcla (combinaciones) de productos (moléculas) conocidos y su variación, bien sea en uso, forma, dimensión o materia, no se consideran invenciones por cuanto no son susceptibles de aplicación industrial, nivel inventivo y novedad.

Finalmente el opositor solicita tener en cuenta los documentos "A novel human monoclonal antibody that binds with high affinity to mesothelin-expressing cells and kills them by antibody-dependent cell-mediated cytotoxicity", "New monoclonal antibodies to mesothelin useful for immunohistochemistry, fluorescence-activated cell sorting, Western blotting, and elisa", y "Preclinical evaluation of MORAb-009, a chimeric antibody targeting tumor-associated mesothelin", cancer immunity, academy of cancer immunology" porque en su concepto los documentos afectan los requisitos de Novedad y/o Nivel Inventivo de la materia revelada en la solicitud.

Respuestas de la Solicitante

La solicitante no da respuesta a los argumentos de la sociedad opositora, pero esta modifica el capítulo reivindicatorio.

Respuesta del Examinador Técnico

Frente a los documentos aportados por la sociedad opositora este Despacho considera que:

1. El documento "A novel human monoclonal antibody that binds with high affinity to mesothelin-expressing cells and kills them by antibody-dependent cell-mediated cytotoxicity" divulga anticuerpos similares a la solicitud que presentan valores de afinidad similares a los de la presente solicitud.
2. El documento "New monoclonal antibodies to mesothelin useful for immunohistochemistry, fluorescence-activated cell sorting, Western blotting, and ELISA", divulga anticuerpos anti-mesotelina útiles como agentes de diagnóstico del cáncer.
3. El documento "Preclinical evaluation of MORAb-009, a chimeric antibody targeting tumor-associated mesothelin", cancer immunity, academy of cancer immunology", enseña anticuerpos estructuralmente similares a los de la solicitud.

En conclusión los documentos presentados en la oposición desvirtúan el nivel inventivo del objeto de esta solicitud; por lo cual se considerarán dentro del examen de patentabilidad.

8. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: 20 de Diciembre del 2010, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	Clinical cancer research, the american association for cancer research, us, vol. 11, no. 16	"A novel human monoclonal antibody that binds with high affinity to mesothelin-expressing cells and kills them by antibody-dependent cell-mediated cytotoxicity"	15/Agosto/2005
D2	The american association for cancer research, us, vol. 11, no. 16	"New monoclonal antibodies to mesothelin useful for immunohistochemistry, fluorescence-activated cell sorting, Western blotting, and ELISA",	15/Agosto/2005
D3	Cancer immunity, academy of cancer immunology, ch, vol. 7	"Preclinical evaluation of MORAb-009, a chimeric antibody targeting tumor-associated mesothelin"	16/Diciembre/2007
D4	WO2009045957	"Human antibodies that bind mesothelin, and uses thereof"	09/Abril/2009
D5	Bioconjugate Chem. 2010, 21, 5-13	"Antibody-Drug Conjugates: Linking Cytotoxic Payloads to Monoclonal Antibodies"	07/Julio/2009

El documento D1¹ divulga un anticuerpo monoclonal que se une a la mesotelina y su unión no se afecta por la glicosilación, con valores de afinidad de de 1.5nM, donde este anticuerpo presenta (Pág. 1114):

Una cadena ligera definida por los siguientes aminoácidos (Pág. 1114):

```
DIQMTQSPSS LSASVGDRVT ITCRASQSI SYLNWYQQKP GKAPKLLIYA
ASSLQSGVPS GFSGSGSGTD FTLTISSLQP EDFATYYCQQ SYSTPLTFGG
GTKVEIKRTV AAPSVFIFPP SDEQLKSGTA SVCLLNIFY PREAKVQWKV
DNALQSGNSQ ESVTEQDSKD STYLSSTLT LSKADYEKHK VYACEVTHQG
LSSPVTKSFN RGE
```

Una cadena pesada definida por los siguientes aminoácidos (Pág. 1114):

```
QVQLQESGPG LVKPSETLSL TCTVSGGSVS SGSYYWSWIR QPPGKGLEWI
GYIYYSGSTN YNPSLKSRVT ISVDTSKNQF SLKLSSVTAA DTAVYYCARE
GKNGAFDIWG QGTMVTVSSA STKGPSVFPL APSSKSTSGG TAALGCLVKD
YFPEPVTVSW NSGALTSGVH TFAVLQSSG LYSLSVTV PSSLGTQTY
ICNVNHKPSN TKVDKKVEPK SCDKTSQGAG
```

1. Url: <http://www.ncbi.nlm.nih.gov/pubmed/19417159>

El documento D2² revela anticuerpos monoclonales útiles como agentes inhibidores de la mesotelina, que presentan valores de actividad entre 1,1 y 12 nM (Pág. 5842, Tabla 1).

El documento D3³ divulga un anticuerpo llamado MORAb-009 útil como agente anti-mesotelina, donde este anticuerpo es conocido como amatuximab (Pág. 19).

El documento D4 divulga compuestos conjugados que comprenden anticuerpos anti-mesotelina unidos a linkers que comprenden un agente citotóxico (Pág. 60).

El documento D5 divulga conjugados de anticuerpo-fármaco (ADC) que combinan la especificidad de los anticuerpos monoclonales (mAb) con la potencia de moléculas citotóxicas (Resumen).

9. Examen de Patentabilidad (Art 14 D486)

Nivel inventivo (Art 18 D486)

Nivel inventivo Frente a los documentos D1 y D2.

La reivindicación 8 consiste en: *“Un anticuerpo de cualquiera de las reivindicaciones 1-7 que comprende (a) una secuencia VH de SEQ ID N°8 y una secuencia VL de SEQ ID N°4”* (Radicado N°. 13-135221-00004-0000).

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Característica s esenciales	D1	D3
Un anticuerpo anti-mesotelina	Un anticuerpo anti-mesotelina (Pág. 1114)	Anticuerpos monoclonales útiles como agentes inhibidores de la mesotelina, (Pág. 5842, Tabla 1).
Cadena pesada SEQ ID N°8	Una cadena pesada que comprende la secuencia (Pág. 1114) QVQLQESGPG LVKPSETLSL TCTVSGGSVS SGSYYWSWIR QPPGKGLEWI GYIYYSGSTN YNPSLKSRVT ISVDTSKNQF SLKLSSVTAA DTAIVYYCARE GKNGAFDIWG QGTMTVSSA STKGPSVFPL APSSKSTSGG TAALGCLVKD YFPEPVTVSW NSGALTSGVH TFPAVLQSSG LYSLSVVTV PSSSLGTQTY ICNVNHNKPSN TKVDKKVEPK SCDKTSGQAG	
Cadena ligera SEQ ID N°8	Una cadena ligera que comprende la secuencia (Pág. 1114) DIQMTQSPSS LSASVGDRVT ITCRASQSIG SYLNWYQQKP	

2.Url: <http://clincancerres.aacrjournals.org/content/11/16/5840.full.pdf+html>

3.Url: <http://www.ncbi.nlm.nih.gov/pubmed/18088084>

	GKAPKLLIYA ASSLQSGVPS GFSGSGSGTD FTLTISSLQP EDFATYYCQQ SYSTPLTFGG GTKVEIKRTV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYLSSTLT LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC	
--	--	--

El documento D1 se considera el estado de la técnica más cercano a la invención definida en la reivindicación 8, porque divulga un anticuerpo capaz de unirse a la mesotelina y su unión no se afecta por la glicosilación, con valores de afinidad de 1.5nM (Pág. 1114).

La diferencia entre la invención, definida en la reivindicación 1 y los anticuerpos definidos en la reivindicación 8 de la solicitud corresponde a que los anticuerpos de la solicitud presentan CDRs diferentes a los del documento D1.

No se evidencia un efecto técnico inesperado asociado al cambio de los CDRs en anticuerpo anti-mesotelina, considerando que los valores de afinidad aportados por la sociedad solicitante son mayores o iguales a los divulgados en el documento D1.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: cómo proporcionar anticuerpos ant-mesotelina alternativos a los divulgados en el documento D1.

Sin embargo, la modificación de los CDRs en anticuerpos útiles como agentes inhibidores de la mesotelina, ya está divulgada en el documento D2 que se refiere a un anticuerpo anti-mesotelina conocido como amatuximab que presenta modificaciones en los CDRs respecto al anticuerpo anti-mesotelina natural, dando como resultado mejoras en la afinidad sobre la mesotelina y valores similares a los presentados por la solicitud (Abstract).

En consecuencia, la persona del oficio normalmente versada en la materia, incluiría modificaciones en los CDRs como las divulgadas del documento D2 en los anticuerpos divulgados en el documento D1 para llegar así al objeto de la reivindicación 8 de la solicitud. Por lo cual se considera obvia.

Puesto que las reivindicaciones dependientes 1-7 y 9-26 no mencionan características inventivas adicionales tampoco se pueden considerar inventivas.

Conclusión:

Las reivindicaciones 1 -26 cumplen el requisito de novedad (Art. 16), pero no cumplen con el requisito de nivel inventivo (Art. 18).

La reivindicación 8 consiste en: *“Un anticuerpo de cualquiera de las reivindicaciones 1-7 que comprende (a) una secuencia VH de SEQ ID N°8 y una secuencia VL de SEQ ID N°4”* (Radicado N°. 13-135221-00004-0000).

Nivel inventivo Frente a los documentos D1 y D3.

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Característica s esenciales	D1	D3
Un anticuerpo anti-mesotelina	Un anticuerpo anti-mesotelina (Pág. 1114)	Un anticuerpo anti-mesotelina (Pág. 19).
Cadena pesada SEQ ID N°8	Una cadena pesada que comprende la secuencia (Pág. 1114) QVQLQESGPG LVKPSETLSL TCTVSGGSVS SGSYYWSWIR QPPGKGLEWI GYIYYSGSTN YNPSLKSRTV ISVDTSKNQF SLKLSSVTAA DTAVYYCARE GKNGAFDIWG QGTMVTVSSA STKGPSVFPL APSSKSTSGG TAALGCLVKD YFPEPVTVSW NSGALTSGVH TFPAVLQSSG LYSLSVVTV PSSSLGTQTY ICNVNHKPSN TKVDKKVEPK SCDKTSGQAG	Una cadena pesada que comprende la secuencia (Pág. 19). QVQLQSGPE LEKPGASVKI SCKASGYSFT GYTMNWKQS HGKSLEWIGL ITPYNGASSY NQKFRGKATL TVDKSSSTAY MDLLSLTSED SAVYFCARGG YDGRGFDYWG SGTPTVTSSA STKGPSVFPL APSSKSTSGG TAALGCLVKD YFPEPVTVSW NSGALTSGVH TFPAVLQSSG LYSLSVVTV PSSSLGTQTY ICNVNHKPSN TKVDKKVEPK SCDKTHTCPP CPAPELLGGP SVFLFPPKPK DTLMISRTP E VTCVVVDVSH EDPEVKFNWY VDGVEVHNAK TKPREEQYNS TYRVVSVLTV LHQDWLNGKE YKCKVSNKAL PAPIEKTISK AKGQPREPQV YTLPPSRDEL TKNQVSLTCL VKGFYPSDIA VEWESNGQPE NNYKTTTPVL DSDGSFFLYS KLTVDKSRWQ QGNVFSCSVM HEALHNHYTQ KSLSLSLSPGK
Cadena ligera SEQ ID N°8	Una cadena ligera que comprende la secuencia (Pág. 1114) DIQMTQSPSS LSASVGDRVT ITCRASQSIG SYLNWYQQKP GKAPKLLIYA ASSLQSGVPS GFGSGSGSTD FTLTISSLQP EDFATYYCQQ SYSTPLTFTGG GTKVEIKRTV AAPSVFIFPP SDEQLKSGTA SVVCLLNIFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYLSSTLT LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC	Una cadena ligera que comprende la secuencia (Pág. 19). DIELTQSPAI MSASPGKVT MTCSASSSVS YMHWYQKSG TSPKRWIYDT SKLASGVPR FSGSGSGNSY SLTISSVEAE DDATYYCQW SKHPLTFGSG TKVEIKRTVA APSVFIFPPS DEQLKSGTAS VVCLLNIFYP REAKVQWKVD NALQSGNSQE SVTEQDSKDS TYSLSSTLT SKADYEKHKV YACEVTHQGL SSPVTKSFNR GEC

El documento D1 se considera el estado de la técnica más cercano a la invención definida en la reivindicación 8, porque divulga un anticuerpo capaz de unirse a la mesotelina y su unión no se afecta por la glicosilación, con valores de afinidad de 1.5nM (Pág. 1114).

La diferencia entre la invención, definida en la reivindicación 1 y los anticuerpos definidos en la reivindicación 8 de la solicitud corresponde a que los anticuerpos de la solicitud presentan CDRs diferentes a los del documento D1.

No se evidencia un efecto técnico inesperado asociado al cambio de los CDRs en anticuerpo anti-mesotelina, considerando que los valores de afinidad aportados por la sociedad solicitante son mayores o iguales a los divulgados en el documento D1.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: cómo proporcionar anticuerpos ant-mesotelina alternativos a los divulgados en el documento D1.

Sin embargo, la modificación de los CDRs en anticuerpos útiles como agentes inhibidores de la mesotelina, ya está divulgada en el documento D3 que se refiere a un

anticuerpo que presenta modificaciones en los CDRs respecto al anticuerpo anti-mesotelina natural, dando como resultado mejoras en la afinidad sobre la mesotelina y valores similares a los presentados por la solicitud (Abstract y Tabla 1).

En consecuencia, la persona del oficio normalmente versada en la materia, incluiría modificaciones en los CDRs como las divulgadas del documento D3 en los anticuerpos divulgados en el documento D1 para llegar así al objeto de la reivindicación 8 de la solicitud. Por lo cual se considera obvia.

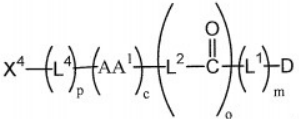
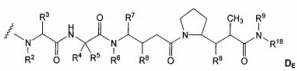
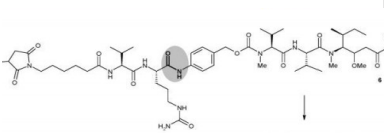
Puesto que las reivindicaciones dependientes 1-7 y 9-26 no mencionan características inventivas adicionales tampoco se pueden considerar inventivas.

Conclusión:

Las reivindicaciones 1 -26 cumplen el requisito de novedad (Art. 16), pero no cumplen con el requisito de nivel inventivo (Art. 18).

La reivindicación 28 consiste en: “Un inmunoconjugado que posee la fórmula AB-(L-D)p (...)” (Radicado N°. 13-135221-00004-0000).

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D5	D4
Un inmunoconjugado	Un inmunoconjugado	Un inmunoconjugado
Posee la fórmula Ab-(L-D)p	Posee la fórmula Ab-(L-D)p (Tabla 1)	Posee la fórmula (I) (Pág. 66-78) 
AB es un anticuerpo-anti-mesotelina	AB es un anticuerpo monoclonal (Tabla 1)	X4 es un anticuerpo anti-mesotelina (Pág. 66-78)
L es una unión	L es un dipéptido (Tabla 1)	AA1es un dipéptido (Pág. 66-78)
D es un fármaco  R2 y R6 metilo, R3 y R4 isopropilo, R5 es H, R7 sec-butilo, R8 metilo		D es una molécula peptídica (Pág. 66-78)
P es 1 a 8	Unión n 1 o más (Tabla 1)	Con diferentes uniones al anticuerpo (Pág. 66-78)

El documento D4 divulga conjugados de anticuerpo-fármaco (ADC) que combinan la especificidad de los anticuerpos monoclonales (mAb) con la potencia de moléculas citotóxicas (Resumen).

La diferencia entre la invención, definida en la reivindicación 1 y los conjugados definidos en la reivindicación 28 de la solicitud corresponde a que los anticuerpos unidos a los conjugado de la solicitud presentan actividad anti-mesotelina.

El hecho de incluir un anticuerpo anti-mesotelina en conjugado permite que la molécula sea capaz de inhibir la mesotelina.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: cómo proporcionar conjugados con actividad frente a la mesotelina.

Sin embargo, la inclusión de anticuerpos monoclonales anti-mesotelina en conjugados que comprenden conjugados, ya está divulgada en el documento D4 que se refiere a

conjugados que comprenden moléculas citotóxicas junto a anticuerpo anti-mesotelina, dando como resultado inhibición de la mesotelina.

En consecuencia, la persona del oficio normalmente versada en la materia, incluiría anticuerpos monoclonales con actividad anti-mesotelina del documento D4 en los conjugados del documento D3 para llegar así al objeto de la reivindicación 28 de la solicitud. Por lo cual se considera obvia.

Puesto que las reivindicaciones dependientes 29 a 36 no mencionan características inventivas adicionales tampoco se pueden considerar inventivas.

Conclusión:

Las reivindicaciones 28 -39 cumplen el requisito de novedad (Art. 16), pero no cumplen con el requisito de nivel inventivo (Art. 18).

10. Conclusión

Los requisitos de Patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	Clinical cancer research, the american association for cancer research, us, vol. 11, no. 16	1-27	Nivel inventivo
D2	The american association for cancer research, us, vol. 11, no. 16	1-27	Nivel inventivo
D3	Cancer immunity, academy of cancer immunology, ch, vol. 7	1-27	Nivel inventivo
D4	WO2009045957	28-36	Nivel inventivo
D5	Bioconjugate Chem. 2010, 21, 5–13	28-36	Nivel inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir del día siguiente de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2015-05-19

Elaboró: Diego Armando Galvis Sanchez
Revisó: Dayron Alexander Ramirez Reyes

Published in final edited form as:

Mol Cancer Ther. 2009 May ; 8(5): 1113–1118. doi:10.1158/1535-7163.MCT-08-0945.

A Novel Human Monoclonal Antibody that Binds with High Affinity to Mesothelin-Expressing Cells and Kills them by ADCC

Yang Feng^{1,*}, Xiaodong Xiao¹, Zhongyu Zhu^{1,2}, Emily Streaker¹, Mitchell Ho³, Ira Pastan³, and Dimitre S. Dimitrov^{1,*}

¹Protein Interactions Group, CCRNP, NCI-Frederick, NIH, Frederick, MD 21702

²BRP, SAIC-Frederick, Inc., NCI-Frederick, Frederick, MD 21702

³Laboratory of Molecular Biology, CCR, NCI, NIH, Bethesda, MD 20892

Abstract

Mesothelin is a potential new target for cancer immunotherapy because it is present at relatively low levels only in mesothelial cells of pleura, peritoneum and pericardium of healthy people but is significantly elevated in a number of tumors, including mesothelioma, ovarian, pancreatic and lung cancers. However, all currently available antibodies against mesothelin are either murine or chimeric which could limit their use because of increased likelihood of immunogenicity compared to fully human antibodies. Here we report the identification and characterization of a novel fully human monoclonal antibody, m912, which was isolated from a human Fab library by panning against recombinant mesothelin. This antibody in scFv, Fab and IgG1 formats bound specifically and with high affinity (equilibrium dissociation constant in the nM range) to cell surface-associated human mesothelin and to recombinant mesothelin. It specifically lysed cancer cells engineered to express mesothelin in the presence of peripheral blood mononuclear cells isolated from healthy donors most likely by antibody-dependent cellular cytotoxicity (ADCC). M912 is the first reported fully human monoclonal antibody to mesothelin, which has potential for cancer treatment and diagnosis

Keywords

mesothelin; mesothelioma; ovarian cancer; lung cancer; therapeutic antibody

Introduction

Mesothelin was identified as antigen to an antibody Mab K1, which specifically recognized several ovarian cancers (1). It is encoded as a 628-amino acids glycoprotein and cleaved by furin into a membrane-attached 40 kD form, mesothelin, and a smaller form released from cells (2). Mesothelin is attached to cell surface *via* glycosyl-phosphatidyl inositol link to its carboxyl terminus. Presently, limited knowledge about its function is available. Mice with both copies of mesothelin genes inactivated seem to have normal growth and reproduction ability (3). It has been reported that mesothelin interacts to CA125 (or MUC16), an ovarian cancer antigen, and the interaction may play a role in metastasis of ovarian cancers to the peritoneal cavity (4,5). The downstream signals activated by the interaction of CA125 are still not clear.

*Corresponding Authors: Yang Feng, CCRNP, NCI-Frederick, NIH, Bldg 469, Rm 139, Frederick, MD 21702-1201, Phone: 301-846-5629; FAX: 301-846-5598, e-mail: yfeng@ncifcrf.gov, Dimitre S. Dimitrov, CCRNP, NCI-Frederick, NIH, Bldg 469, Rm 150B, Frederick, MD 21702-1201, Phone: 301-846-1352; FAX: 301-846-5598, e-mail: dimitrov@ncifcrf.gov.

The unique distribution pattern of mesothelin in human bodies suggests its potential as a cancer target. In healthy people, mesothelin expression is limited to mesothelial cells lining the pleura, peritoneum and pericardium. Other normal tissues tested do not express mesothelin protein (1). However, mesothelin is over-expressed in a high percentage of ovarian cancers, pancreatic cancers, non-small lung cancers and mesothelioma (6–8). It has been reported that a majority of serous carcinomas of the ovary and adenocarcinomas of the pancreas express high levels of mesothelin (9). In addition, high levels of mesothelin have been detected in >55% of lung cancers and >70% ovarian cancers (7,10). In mesothelioma patients, mesothelin protein is not only readily detectable on tumors, but it is also present in patient serum (11). Furthermore, the mesothelin-positive lung cancer cells die upon exposure to a recombinant immunotoxin targeted to mesothelin (10). Because of its limited distribution in normal tissues and elevated expression in cancers, mesothelin has been considered as an excellent target for cancer therapy. Various methods have been employed to deliver cytotoxic drugs to mesothelin-positive cells or elicit cell-mediated and humoral responses to mesothelin and in turn eliminate tumors. DNA vaccines against mesothelin have been shown to inhibit tumor growth in a mouse model (12,13). A fusion protein of mesothelin-specific single chain and immunotoxin (SS1P) is currently in phase I trial (14). A chimeric monoclonal antibody specific to mesothelin, MORAb-009, is being tested in a phase I trial. In a xenograft model, MORAb-009 synergizes with chemotherapy drugs taxol and gemcitabine, even though it has little effect when used alone in these models (15). Given the potential of targeting mesothelin as an effective treatment for mesothelin-positive tumors, a fully-human therapeutic antibody could provide additional options in terms of immunogenicity and better tolerance. Here we describe a high-affinity fully human mesothelin antibody with a potential as a cancer therapeutic.

Materials and Methods

Cell cultures

A431 cells, human epidermoid carcinoma cells, were maintained in RPMI1640 supplemented with 10% FBS and penicillin/streptomycin (complete growth medium). A431 cells do not express mesothelin. H9 cells were stable clone cells established from A431 cells that have been transfected with a vector carrying full-length mesothelin cDNA. H9 cells were maintained in complete RPMI1640 growth medium supplemented with 0.75 mg/ml G418. OVCAR-3 cells were purchased from ATCC, and maintained in RPMI1640 complete growth medium.

Expression of recombinant mesothelin protein

Human mesothelin fragment including amino acids 296~600 (the numbers are based on sequence in AY743922 in the NCBI database) was cloned from pcDNA3.2 to a baculovirus transfer vector pAcGP67 *via* Sma I and Not I sites. The recombinant product had extra residues ADPG on the N-terminus and 6 histidines on the C-terminus. It was co-transfected with BaculoGold viral DNA into SF9 insect cells according to the manufacturer's instruction. Mesothelin protein was purified from conditioned medium with a nickel-chelating column, and further polished with a Superdex75 gel filtration column in PBS. Purity of mesothelin was examined with SDS-PAGE.

Antibody selection by phage display

Purified mesothelin was labeled with biotin first and used for panning of a human naïve Fab phage library (16). Briefly, amplified phage ($\sim 10^{12}$ pfu) pre-absorbed with MyOne streptavidin T1 beads (Invitrogen) was incubated with 4 μ g of biotin-mesothelin for 2 hr. Specific phages were captured by fresh streptavidin beads. After extensive washes of the beads with PBS+0.05% Tween 20, phage was rescued by exponentially growing TG1

bacteria and helper phage. Pannings were repeated for three more times with more stringent washes at the latter two rounds. Three hundred colonies were picked from the last two rounds of panning and rescued with helper phage for screening. Two unique clones were selected, of which m912 had higher affinity and was selected for further characterization.

Antibody expression and purification

The Fab fragment was expressed in HB2151 cells as described previously (17). A single chain form of m912 was made by cloning VH and VL from Fab, connected by linker 3(GGGGS), into pComb3x (18). Expression and purification of scFv were similar to that of the Fab. Fab was converted into IgG1 by subcloning the heavy chain variable region and the light chain into pDR12. 293 Free Style cells were transfected with pDR12-m912, and IgG was secreted into medium. Fab and IgG were purified with a protein G column, and scFv was purified with a nickel-chelating column. All preparations were dialysed against PBS.

ELISA binding assay

Antigen (mesothelin) diluted in PBS is coated on narrow-well 96 well plate at 50 ng/well overnight at 4°C. Wells are blocked with 100 μ l of 4% milk/PBS (MPBS) for 1 hr at 37°C. For Fab binding kinetics, Fab is titrated from 3000 nM to 0.038 nM (1: 5 serial dilutions). 50 μ l of diluted Fab is added to duplicate wells. In competition ELISA, designated concentration of competing IgG was included in all Fab solutions. After 2 hr incubation at 37°C, the wells are washed with PBST (PBS+0.05% Tween 20) for 4 times. Bound Fab was detected with anti-FLAG-HRP mAb (1:1000) (Sigma) for 1 hr at 37°C. Wells are washed again with PBST. Substrate ABTS is added (50 μ l/well), and the reaction is read at 405 nm. For ELISA with IgG, a goat anti-human Fc IgG conjugated with HRP was used at 1:1000.

Flow cytometry

A413 cells and H9 cells were detached by cell dissociation buffer and rinsed in PBS. Aliquots of cells were incubated with primary antibody (m912, or isotype controls) at indicated concentrations in 250 μ l of RPMI+10% FBS for 1 hr on ice. Unbound antibodies were washed away with medium. Secondary antibody goat anti-human IgG conjugated with FITC (Sigma) was incubated with cells at 8 μ l/ml for 30 min. For detection of Fab or scFv, 1.6 μ g/ml of anti-His6 monoclonal antibody (Qiagen) and 8 μ l/ml of goat anti-mouse IgG-FITC (Sigma) were incubated with cells. Cells were washed and resuspended in PBS+0.5% BSA for flow cytometry on FACSCalibur (Beckton Dickinson).

Cell lysis by ADCC (antibody-dependent cell-mediated cytotoxicity)

Peripheral blood mononuclear cells (PBMC) were isolated from healthy donors with Ficoll-Paque Plus (GE Healthcare). Collections of blood from donors were approved by NCI-Frederick Research Donor Program. The viability of isolated cells was >95%. PBMC were seeded in a 96 well plate in RPMI+10% FBS at 500,000 cells/well. Cells were incubated at 37°C and allowed to attach to the plate for 3 hr. Unattached cells were rinsed off by two washes of warm PBS, cells attached in the wells were used as the effector cells. Target cells, A431 or H9 cells, were trypsinized and resuspended into single cell suspensions. The target cells were incubated with various concentrations of antibody at room temperature for 30 min then added to effector cells at 10,000 cells/well. The ratio of effector and target cells is 50:1. The plate was centrifuged at 300g $\times$ 5 min and incubated at 37°C for 24 hr. Supernatant (100 μ l) was transferred to an all-white plate and 100 μ l of CytoTox-ONE reagent (Promega) was added to each well. The lactate dehydrogenase (LDH) released from lysed cells converted CytoTox substrate to fluorescent resazurin, which was measured in fluorometer (Ex 560 nm/Em 590 nm). The percentage of specific lysis was calculated as following: (experimental treatment-effector cell control)/(high control-target cell control) $\times$ 100%. Measurement of

target cells alone treated with 1% Triton X-100 was used as high control. Each treatment was carried out in 6 duplicated wells. Each assay plate included control wells.

Western blot

H9 and OVCAR-3 cells were lysed in RIPA buffer (50 mM Tris, pH 7.4, 1% Triton X-100, 0.1% SDS, 1% deoxycholate, 150 mM NaCl, 5 mM NaF, 5 mM EDTA). After centrifugation at 20,000 g × 20 minutes, the clear supernatant was resolved on 4~12% NuPAGE and transferred to PVDF membrane. Primary antibodies used for blotting were MORAb-009 at 0.5 ug/ml (for mesothelin) and goat polyclonal antibody against actin (clone I-19, Santa Cruz Biotechnology, for monitoring equal loading) at 1 ug/ml, respectively. Corresponding secondary antibodies were goat anti-human Fc-HRP and donkey anti-goat IgG-HRP at 1 ug/ml for detection.

Results

Expression of recombinant human mesothelin in insect cells and its purification

To efficiently select antibodies from phage libraries a purified recombinant antigen, in this case mesothelin, is needed. In order to include as much as possible of the mesothelin molecule, we expressed its entire extracellular domain, including the GPI linkage site serine-598 (19). To achieve relatively high yields and glycosylation in eukaryotic cells, mesothelin was cloned in a baculovirus expression vector. The transfer vector pAcGP67 has a signal peptide that directs secretion of recombinant protein into medium. Indeed, recombinant mesothelin (296–600 aa) expressed in SF9 insect cells was secreted into the culture medium. The mesothelin purified by a two-step procedure was >95% in purity and migrated at ~35 kD (Figure 1A) on polyacrylamide gel. It migrated slightly faster than the same fragment that was expressed in mammalian cells (data not shown), reflecting differences in post-translational modifications, such as glycosylation. The purified mesothelin was recognized by MORAb-009 (20), a mouse/human chimeric IgG, and was used for panning of a naïve human Fab phage display library.

High-affinity binding of m912 to recombinant mesothelin

Mesothelin produced in insect cells was labeled with biotin and used for panning of our large naïve Fab library as described in Materials and Methods. After the third and fourth rounds of panning, three hundred clones were screened and two positive clones with different sequences were identified. Clone m912 bound with higher affinity to mesothelin than the other clone and was selected for further characterization. Fab m912 was converted to single chain and IgG1 formats. Fab m912 bound to mesothelin with an EC50 of 20 nM as measured by ELISA (Figure 1B). Including of MORAb-009 at a concentration higher than its IC50 in Fab m912 ELISA did not reduce binding signals, suggesting that the two antibodies have different epitopes on mesothelin (Figure 1B). The IgG1 exhibited higher effective binding affinity (avidity) of ~1.5 nM as measured by ELISA (Figure 1C). Deglycosylation of mesothelin by PNGase F reduced its size by at least 3 kD, but it did not change the binding ability of m912 (data not shown), indicating that the m912 epitope is independent of mesothelin glycosylation. Fab m912 also recognized mouse mesothelin with about the same affinity (Figure 1D).

Specific binding of m912 to cell surface-associated mesothelin

It is essential that a therapeutic or diagnostic antibody recognize the native protein. Therefore, Fab m912 was tested for binding to native mesothelin associated with cell surfaces. We used H9 cells which are derived from parental cells (A431), which do not express mesothelin, and stably express mesothelin protein. Fab m912 bound to H9 cells but

not to A431 cells (Figure 2B), indicating that it is highly specific and does not recognize other membrane proteins on these cells.

Various clinical applications require different sizes and valences of antibodies for best effect. For example, small sizes are preferred for targeting and imaging, whereas full-size antibodies (IgGs) have much longer half-life in circulation, and some are able to mediate effector functions. Therefore, Fab m912 was converted to scFv and IgG formats, and tested for binding to cells by using flow cytometry. We found that m912 in both formats, scFv and IgG1, can bind specifically to cell surface-associated mesothelin (Figure 2C and Figure 3A).

To estimate the binding ability (avidity) of IgG1 m912 to native cell surface-associated mesothelin, we used antibody concentrations ranging from 0.1 nM to 3125 nM and H9 cells in a flow cytometry experiment (Figure 3B). Even at the lowest concentration of 0.1 nM IgG m912 exhibited significant binding to H9 cells. Based on the medium fluorescent units bound to H9 cells at each m912 concentration, a 50% binding of m912 was estimated to be at about 5~10 nM for these cells.

The binding of m912 was further tested in non-transfected cancer cells, ovarian cancer cell line, OVCAR-3. These cells have been reported to be positive with cell surface mesothelin. In flow cytometry, m912 showed specific binding in a dose-dependent fashion (Figure 3C); however, higher concentrations were required to reach the same level of binding seen with H9 cells. This was partially due to the much lower levels of mesothelin protein expressed by the OVCAR-3 cells than by H9 cells as demonstrated by the Western blot (Figure 3D). MORAb-009 still showed high avidity on OVCAR-3 cells.

Specific lysis of mesothelin-positive cells by m912 in presence of PBMC

Therapeutic antibodies can kill cancer cells *via* ADCC mediated by the Fc portion of the IgG. To test whether IgG1 m912 has such activity we used a cell growth assay based on A431 and H9 cells. Incubation of IgG1 m912 alone with these cells did not affect their growth (data not shown). However, in the presence of PBMCs isolated from peripheral blood of healthy donors, IgG1 m912 specifically lysed mesothelin-positive (H9) cells likely by ADCC but not mesothelin-negative (A431) cells. The mouse-human chimeric anti-mesothelin antibody MORAb-009 (15) used as a positive control exhibited similar activity, while a control isotype antibody had a baseline lysis activity (Figure 4). These results indicate that IgG1 m912 lysed the H9 cells by ADCC through specific binding to cell surface-associated mesothelin.

Discussion

Mesothelin is an attractive target for cancer therapy because of its differential cell surface-associated expression in normal and tumor cells. It is differentially expressed not only in mesothelioma but also in ovarian, pancreatic and lung cancers (6–8). Although the cellular function of mesothelin is not clear at present, mesothelin-targeted therapies have shown benefits in a number of studies in animal models (12,13) and several early clinical trials are ongoing (14,15). Among thirty-three patients (with mesothelioma, ovarian and pancreatic cancers) treated with SS1P, a recombinant anti-mesothelin immunotoxin, at least two patients had complete resolution of ascites, and 50% of the patients had stable disease (14). In a separate clinical study, pancreatic cancer patients vaccinated with autologous tumor cells have demonstrated increased mesothelin-specific CD8⁺ T cell response, including CD8⁺ T cells that are able to lyse mesothelin-expressing tumor cells (21). These encouraging clinical results support the notion of targeting certain types of cancer cells through mesothelin and justify further studies on mesothelin therapeutics. A fully human mesothelin antibody seems to be a promising addition to mesothelin-related therapeutics

currently under studies. In addition, it has been reported that mesothelin may also be released into serum through deletion at its carboxyl terminus or by proteolytical cleavage from its membrane bound form (19). An increase in the soluble form of mesothelin could be detected several years before malignant mesotheliomas occur among workers exposed to asbestos (22). Patients with ovarian, pancreatic, and lung cancers also have elevated soluble mesothelin in serum (11,23,24). Thus, mesothelin could also be a valuable biomarker for diagnosis and prognosis. New antibodies to mesothelin, therefore, could be also used for detection of mesothelin.

The antibody we have developed, m912, is fully human and in Fab, scFv and IgG formats was able to specifically recognize cell surface-associated human mesothelin. It also bound to mouse mesothelin which would facilitate its use in mouse models of cancer. We have not localized its epitope. However, m912 and MORAb-009 do not compete for binding to mesothelin (Figure 1B) and MORAb-009 does not recognize mouse mesothelin (15), suggesting that m912 binds to an epitope that does not overlap the MORAb-009 epitope. In addition, the cross-reactivity of m912 to human and mouse mesothelin provides an advantage compared to MORAb-009 because it could be used in mouse models.

MORAb-009, which was derived from a mouse immune library and further improved by in vitro maturation, binds with high avidity even to non-transfected cells, such as OVCAR-3, which express relatively low levels of mesothelin (15). M912 was selected from a non-immune human library and was not further matured in vitro. This is why it was not unexpected that its binding to OVCAR-3 cells requires higher concentrations than MORAb-009 to reach the same antibody surface concentration (Fig. 3C). Currently, we are improving the m912 affinity by in vitro maturation. As mentioned above MORAb-009 does not bind to mouse mesothelin and may not be used in mouse models of cancer. In addition, it binds to an epitope which does not overlap the m912 epitope. Thus the two antibodies may have different areas of application and could be used in combination.

The m912 was selected from a naïve library and its sequence does not deviate significantly from the germ line sequences. Its VL and JL regions together have only one mutation compared with the closest germ line gene IGKV1-39 (accession number X59315) and IGKJ4 (J00242). The heavy chain V gene of m912 is identical to the germ line gene IGHV4-61 (M29811) and its J gene is identical to IGHJ3*02 (X86355). The D gene region has two mutations and an N2 insertion compared with IGHD3-16*01 ([X93614](#)).

In the presence of PBMC, IgG1 m912 specifically lysed mesothelin-positive cells likely by ADCC but did not affect mesothelin-negative cells. Thus, even non-conjugated m912 could be a potential candidate therapeutic for treatment of mesothelin-positive tumors. Its tumor killing activity could be further improved by conjugation to toxins or small molecule drugs. The antibody could also be utilized for guiding liposomes or other carriers of anti-cancer drugs or imaging agents to cancer cells, and as an agent for detection of soluble mesothelin in serum of cancer patients.

Abbreviations

ADCC	antibody-dependent cell-mediated cytotoxicity
Fab	antigen-binding fragment
scFv	single chain variable fragment
kD	kilodalton
ELISA	enzyme-linked immunosorbent assay

Acknowledgments

We thank members of our group for helpful discussions. This project was supported by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research, and by federal funds from the National Cancer Institute, NIH, under contract N01-CO-12400.

References

1. Chang K, Pastan I, Willingham MC. Isolation and characterization of a monoclonal antibody, K1, reactive with ovarian cancers and normal mesothelium. *Int J Cancer* 1992;50:373–381. [PubMed: 1735605]
2. Chang K, Pastan I. Molecular cloning of mesothelin, a differentiation antigen present on mesothelium, mesotheliomas, and ovarian cancers. *Proc Natl Acad Sci U S A* 1996;93:136–140. [PubMed: 8552591]
3. Bera TK, Pastan I. Mesothelin is not required for normal mouse development or reproduction. *Mol Cell Biol* 2000;20:2902–2906. [PubMed: 10733593]
4. Rump A, Morikawa Y, Tanaka M, et al. Binding of ovarian cancer antigen CA125/MUC16 to mesothelin mediates cell adhesion. *J Biol Chem* 2004;279:9190–9198. [PubMed: 14676194]
5. Gubbels JA, Belisle J, Onda M, et al. Mesothelin-MUC16 binding is a high affinity, N-glycan dependent interaction that facilitates peritoneal metastasis of ovarian tumors. *Mol Cancer* 2006;5:50. [PubMed: 17067392]
6. Argani P, Iacobuzio-Donahue C, Ryu B, et al. Mesothelin is overexpressed in the vast majority of ductal adenocarcinomas of the pancreas: identification of a new pancreatic cancer marker by serial analysis of gene expression (SAGE). *Clin Cancer Res* 2001;7:3862–3868. [PubMed: 11751476]
7. Hassan R, Kreitman RJ, Pastan I, Willingham MC. Localization of mesothelin in epithelial ovarian cancer. *Appl Immunohistochem Mol Morphol* 2005;13:243–247. [PubMed: 16082249]
8. Li M, Bharadwaj U, Zhang R, et al. Mesothelin is a malignant factor and therapeutic vaccine target for pancreatic cancer. *Mol Cancer Ther* 2008;7:286–296. [PubMed: 18281514]
9. Yen MJ, Hsu CY, Mao TL, et al. Diffuse mesothelin expression correlates with prolonged patient survival in ovarian serous carcinoma. *Clin Cancer Res* 2006;12:827–831. [PubMed: 16467095]
10. Ho M, Bera TK, Willingham MC, et al. Mesothelin expression in human lung cancer. *Clin Cancer Res* 2007;13:1571–1575. [PubMed: 17332303]
11. Cristaudo A, Foddis R, Vivaldi A, et al. Clinical significance of serum mesothelin in patients with mesothelioma and lung cancer. *Clin Cancer Res* 2007;13:5076–5081. [PubMed: 17785560]
12. Chowdhury PS, Pastan I. Analysis of cloned Fvs from a phage display library indicates that DNA immunization can mimic antibody response generated by cell immunizations. *J Immunol Methods* 1999;231:83–91. [PubMed: 10648929]
13. Chang CL, Wu TC, Hung CF. Control of human mesothelin-expressing tumors by DNA vaccines. *Gene Ther* 2007;14:1189–1198. [PubMed: 17581599]
14. Hassan R, Bullock S, Premkumar A, et al. Phase I study of SS1P, a recombinant anti-mesothelin immunotoxin given as a bolus I.V. infusion to patients with mesothelin-expressing mesothelioma, ovarian, and pancreatic cancers. *Clin Cancer Res* 2007;13:5144–5149. [PubMed: 17785569]
15. Hassan R, Ebel W, Routhier EL, et al. Preclinical evaluation of MORAb-009, a chimeric antibody targeting tumor-associated mesothelin. *Cancer Immun* 2007;7:20. [PubMed: 18088084]
16. Zhu ZY, Dimitrov DS. Construction of a large naïve human phage-displayed Fab library through one-step cloning. *Meth Mol Biol*. in press
17. Feng Y, Zhu Z, Xiao X, Choudhry V, Barrett JC, Dimitrov DS. Novel human monoclonal antibodies to insulin-like growth factor (IGF)-II that potently inhibit the IGF receptor type I signal transduction function. *Mol Cancer Ther* 2006;5:114–120. [PubMed: 16432169]
18. Scott, JK.; Barbas, CF, III. Phage-display vector. In: Barbas, CF., III, editor. *Phage display: a laboratory manual*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press; 2001.
19. Hassan R, Bera T, Pastan I. Mesothelin: a new target for immunotherapy. *Clin Cancer Res* 2004;10:3937–3942. [PubMed: 15217923]

20. Chowdhury PS, Viner JL, Beers R, Pastan I. Isolation of a high-affinity stable single-chain Fv specific for mesothelin from DNA-immunized mice by phage display and construction of a recombinant immunotoxin with anti-tumor activity. *Proc Natl Acad Sci U S A* 1998;95:669–674. [PubMed: 9435250]
21. Thomas AM, Santarsiero LM, Lutz ER, et al. Mesothelin-specific CD8(+) T cell responses provide evidence of in vivo cross-priming by antigen-presenting cells in vaccinated pancreatic cancer patients. *J Exp Med* 2004;200:297–306. [PubMed: 15289501]
22. Creaney J, Robinson BW. Detection of malignant mesothelioma in asbestosexposed individuals: the potential role of soluble mesothelin-related protein. *Hematol Oncol Clin North Am* 2005;19:1025–1040. [PubMed: 16325121]
23. Hassan R, Remaley AT, Sampson ML, et al. Detection and quantitation of serum mesothelin, a tumor marker for patients with mesothelioma and ovarian cancer. *Clin Cancer Res* 2006;12:447–453. [PubMed: 16428485]
24. Corso CD, Stubbs DD, Lee SH, Goggins M, Hruban RH, Hunt WD. Real-time detection of mesothelin in pancreatic cancer cell line supernatant using an acoustic wave immunosensor. *Cancer Detect Prev* 2006;30:180–187. [PubMed: 16647225]

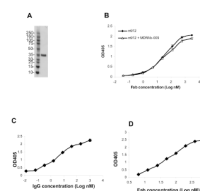


Figure 1. High affinity binding of Fab m912 and IgG1 m912 to recombinant mesothelin

A, A sample of recombinant human mesothelin purified from insect cell supernatant was run on 4~12% NuPAGE Bis-Tris gel. Molecular weight standards are in kD. **B**, Binding of Fab m912 to recombinant mesothelin coated on ELISA plate (-◆-). In competition ELISA, 3 nM MORAb-009 was added to all Fab m912 solutions (-Δ-) **C**, Binding of IgG1 m912 to recombinant mesothelin on ELISA. **D**, Binding of Fab m912 to mouse recombinant mesothelin on ELISA.

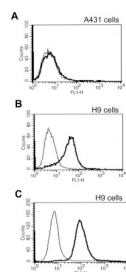


Figure 2. Specific, high-affinity binding of Fab m912 and scFv m912 to cell surface-associated mesothelin

A, Lack of Fab m912 binding to mesothelin-negative (A431) cells. **B**, Binding of Fab m912 but not of a control Fab to mesothelin-positive H9 cells. **C**, Binding of scFv m912 but not of a control scFv to mesothelin-positive H9 cells. In all three panels, thick lines represent binding of m912 in Fab or scFv formats and thin lines are for control Fab and scFv. The concentrations of all Fabs and scFv are 40 nM and 100 nM, respectively.

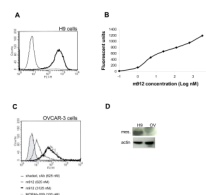


Figure 3. High avidity binding of IgG1 m912 to cell surface-associated mesothelin

A. IgG1 m912 at 5 nM was incubated with mesothelin-positive H9 cells, the cells were washed and further incubated with goat anti-human IgG conjugated with FITC, then washed and analyzed by flow cytometry. An isotype control IgG (thin line) at 100 nM was used as negative control. **B.** The same flow cytometry analyses were performed at different concentrations of IgG1m912, and medium fluorescent units plotted as function of the antibody concentration. **C.** IgG1 m912 at 625 nM (thin line without shade) and 3125 nM (thick line) was incubated with OVCAR-3 cancer cells, as performed similarly in panel A. MORAb-009 was used at 100 nM (dotted line). An isotype control human IgG was used at 625 nM (thin line, shaded area). **D.** Levels of mesothelin proteins in H9 and OVCAR-3 cells as shown by Western blot. Equal amount of lysates were loaded as shown by actin blot.

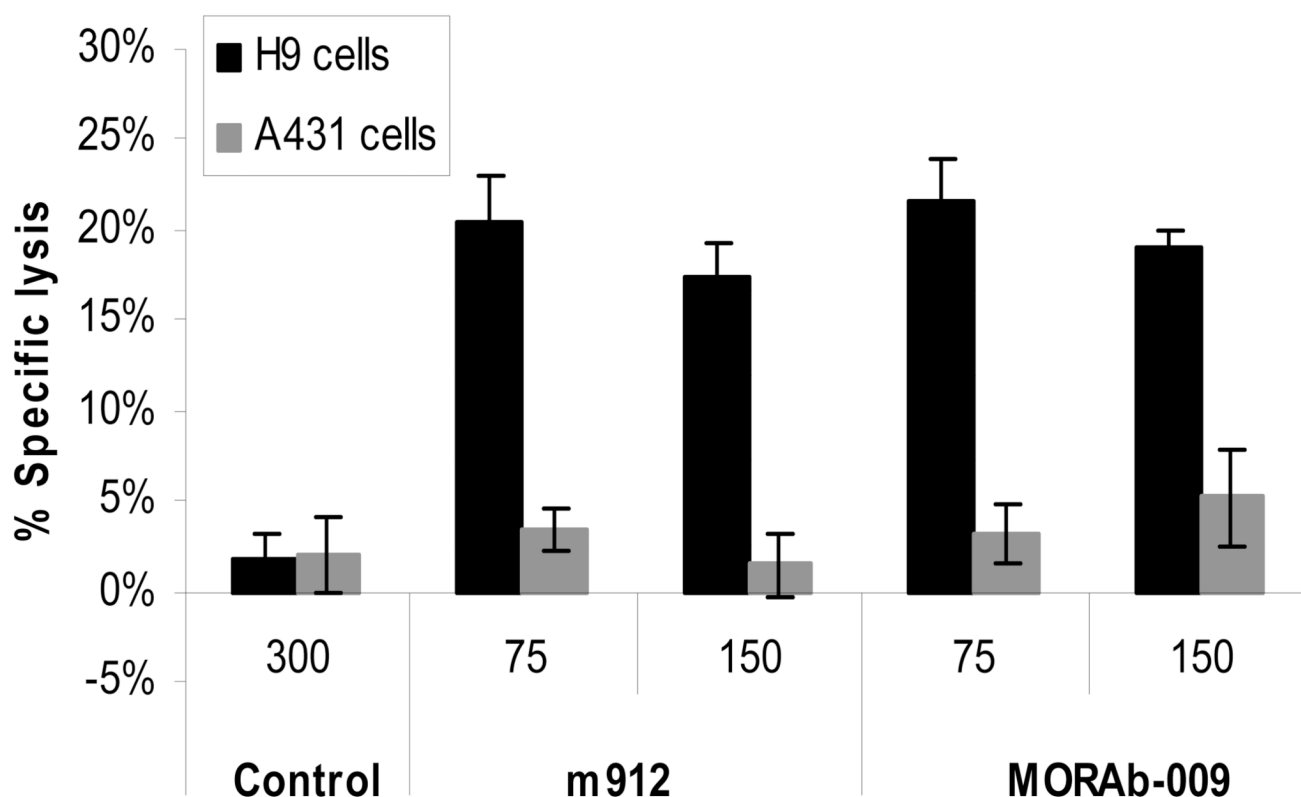


Figure 4. IgG1 m912 induced ADCC in mesothelin-positive cells, but not in mesothelin-negative cells

Freshly isolated PBMCs were incubated with target cells (H9 or A431) at ratio of 50:1, in the presence of IgG1 m912 or MORAb-009 at two different concentrations, 75 or 150 nM, or an isotype control IgG1 at 300 nM. ADCC was detected with CytoTox-ONE reagent, which measures the lactate dehydrogenase released by lysed target cells. The percentage (%) of specific lysis was calculated as described in the Materials and Methods.

New Monoclonal Antibodies to Mesothelin Useful for Immunohistochemistry, Fluorescence-Activated Cell Sorting, Western Blotting, and ELISA

Masanori Onda,¹ Mark Willingham,² Satoshi Nagata,¹ Tapan K. Bera,¹ Richard Beers,¹ Mitchell Ho,¹ Raffit Hassan,¹ Robert J. Kreitman,¹ and Ira Pastan¹

Abstract **Purpose:** Mesothelin is a cell surface protein that is highly expressed in some malignant tumors, and is a promising target for immunotherapy. Recent data suggests that mesothelin is an adhesive protein and may have a role in the metastases of ovarian cancer. Although a few monoclonal antibodies (MAb) to mesothelin have been produced, they have limitations for the study of expression of native mesothelin because of their low affinity or reactivity only with denatured mesothelin protein. We have produced novel MAbs to mesothelin to help study mesothelin function and to develop improved diagnosis and immunotherapy of mesothelin-expressing tumors.

Experimental Design: Mesothelin-deficient mice were immunized with plasmid cDNA encoding mesothelin, and boosted with a mesothelin-rabbit IgG Fc fusion protein prior to cell fusion. Hybridomas were screened by an ELISA using plates coated with mesothelin-Fc protein.

Results: Seventeen hybridomas producing anti-mesothelin antibodies were established and shown to react with two epitopes on mesothelin. One group reacts with the same epitope as the low affinity antibody K1 that was originally used to identify mesothelin. The other is a new group that reacts with a new epitope. One antibody from each group was chosen for further study and shown to react strongly on ELISA, on immunohistochemistry, and by fluorescence-activated cell sorting on living cells.

Conclusion: Our two newly established MAbs, MN and MB, have different and useful properties compared with current antibodies used for the detection of mesothelin by immunohistochemistry, fluorescence-activated cell sorting, ELISA, and Western blotting.

Mesothelin is a 40-kDa glycosylphosphatidylinositol-linked cell surface glycoprotein present on normal mesothelial cells that is highly expressed in mesothelioma, ovarian cancer, pancreatic cancer, and some other malignancies (1–4). The normal biological function of mesothelin is unknown and mesothelin-deficient mice have no phenotype (5). However, a recent report indicates that mesothelin can bind to CA125/MUC16, suggesting that mesothelin might have a role in the metastatic spread of ovarian cancer (6). Initial studies of mesothelin expression were done by immunohistochemistry with monoclonal antibody (MAb) K1 generated by immunization of mice with the human ovarian carcinoma cell line OVCAR-3 (1, 2, 4). These studies showed that mesothelin is highly expressed in many human cancers including ovarian

cancers, mesotheliomas, and squamous cell cancers. The studies were done on frozen sections, because MAb K1 did not work well on fixed tissues (7). However, using antigen retrieval by incubating tissue sections in 3 mol/L urea, mesothelin expression can be detected in paraffin-embedded formalin-fixed tissues. Subsequently, MAb 5B2 was generated by immunizing mice with a recombinant prokaryotic fusion protein corresponding to 100 amino acids which are present in the NH₂ terminus of mesothelin (8). Several immunohistochemical studies have been done with MAb 5B2 (9, 10). The studies confirmed that mesothelin is expressed in mesotheliomas, ovarian cancers, and squamous cell tumors and also showed expression in pancreatic cancer and lung cancer, although the distribution in lung cancer was diffuse and not limited to the plasma membrane. No expression of mesothelin was observed in lung cancer using MAb K1 (1). Because of its high expression in cancers and limited expression on normal tissues, mesothelin is also a promising target for cancer immunotherapy. Immunotoxins are chimeric proteins composed of the Fv portion of an antibody fused to a 38 kDa fragment of *Pseudomonas* exotoxin A (PE38). One of these, BL22, targets the cell surface protein, CD22, and has produced many complete remissions in drug-resistant Hairy Cell Leukemia showing that these agents can be useful in treating cancers in humans (11). We prepared immunotoxin SS1 (dsFv)-PE38 (SS1P) composed of an antibody fragment reacting with

Authors' Affiliations: ¹Laboratory of Molecular Biology, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, Maryland and ²Department of Pathology, Wake Forest University School of Medicine, Winston-Salem, North Carolina Received 3/15/05; revised 5/10/05; accepted 5/26/05.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Ira Pastan, Laboratory of Molecular Biology, National Cancer Institute, 37 Convent Drive, Room 5106, Bethesda, MD 20892-4264. Phone: 301-496-4797; Fax: 301-402-1344; E-mail: pastani@mail.nih.gov.

©2005 American Association for Cancer Research.

doi:10.1158/1078-0432.CCR-05-0578

mesothelin and PE38 for the treatment of mesothelin-expressing cancers. SS1P has been shown to specifically kill mesothelin-expressing cell lines and to cause regressions of mesothelin-expressing tumors in mice (12, 13). Based on these studies and appropriate safety data, two phase 1 trials with SS1P are being conducted at the National Cancer Institute in patients with mesothelin-expressing cancers (14, 15). In addition, other therapies targeting mesothelin are in preclinical development (16).

Hellstrom and colleagues developed an ELISA-based assay to detect a protein in the blood they term soluble mesothelin-related protein (17). The exact relationship of this protein to mesothelin is currently not clear. Interestingly, soluble mesothelin-related protein is elevated in many patients with mesothelioma and some patients with ovarian cancer (18).

Research in the mesothelin area has been hampered by the lack of well-characterized, readily available antibodies that could be used for immunohistochemistry on fixed tissues, Western blotting, fluorescence-activated cell sorting (FACS) analysis of cells from patients, and ELISA to measure mesothelin in the blood and body fluids and other purposes. We describe here two new antibodies reacting with different epitopes on mesothelin that can be used for all these applications.

Materials and Methods

Generation of a mesothelin-Fc fusion protein by mammalian cells. The extracellular domain of the human mesothelin was expressed as a fusion protein with rabbit IgG Fc in HEK 293T cells. The DNA fragment encoding rabbit IgG Fc was amplified by PCR using the plasmid pYB1-12,14 for the RfC (amino acids 96-323, Swissprot, kindly provided by Dr. Rose G. Mage, NIH, ref. 19) as the template and inserted between *Sfi*I and *Sac*II sites of pSecTag2 (Invitrogen, Carlsbad, CA). cDNA for the extracellular domain of mesothelin was inserted between the *Sac*II and *Not*I to obtain the plasmid pOND-rFc-Meso. Primers used were as follows: Meso forward; 5'-AGA TAG AGT CCG CGG GGA GGT GAA GTG GAG AAG ACA GCC TGT-3', Meso reverse; 5'-TTG TAT AGC GGC CGC TCA TCC CCC CGA GAG GGC CTC TTG CAC-3'. The plasmid was transfected into 293T cells by LipofectAMINE reagent (Invitrogen). The mesothelin-Fc protein harvested from the culture supernatant and purified with Hi-trap protein A column (Amersham Biosciences Corp., Piscataway, NJ). The purified proteins were quantitated by Coomassie blue (Pierce, Rockford, IL) and checked on SDS-PAGE gel.

Generation of a mesothelin protein by *Escherichia coli*. The mesothelin gene (GenBank accession number AY743922) was cloned from the IMAGE cDNA clone ID 5209096 using PCR and put it into pMAL-p2X (NEB, Beverly, MA) which has a tobacco etch virus protease cleavage site. The resulting plasmid pMH103 encodes a fusion protein consisting of the malE signal sequence, malE and mesothelin. The malE gene encodes the maltose-binding protein. The fusion protein is directed to the periplasm of *Escherichia coli*. The secreted proteins were separated with recombinant tobacco etch virus (Invitrogen). The samples were applied to an amylase column for removal of maltose-binding protein. The purity of bacterial mesothelin was >95%.

Cells. The pancreatic cancer cell line Panc 3.014 was obtained from Dr. Elizabeth Jaffee (Johns Hopkins Medical Institute, Baltimore, MD; ref. 16). It was maintained in RPMI 1640 supplemented with 20% fetal bovine serum, 200 μ mol/L L-glutamine, 50 units/mL penicillin, 100 μ g/mL streptomycin, 1% nonessential amino acids, 1% sodium pyruvate, and 2 units/mL human insulin. The human mesothelioma cells NCI-H226 was obtained from Dr. Isaiah J. Fidler (The University of Texas MD Anderson Cancer Center, Houston, TX). It was maintained in modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, and 1% nonessential amino acids. The human ovarian carcinoma cell line

A1847 and human cervical carcinoma cell line HeLa were grown in DMEM supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 μ g/mL streptomycin.

Immunization. Female mesothelin-deficient mice (6-8 weeks old) were immunized four times with the pcDNA3CAK1-9 plasmid intradermally (50 μ g in 50 μ L PBS) with 2-week intervals, and one boost was given with the mesothelin-Fc i.p. (50 μ g in 100 μ L of PBS) before fusion (5). Spleens were harvested 84 to 90 hours after the last boost for cell fusion. All animals were maintained in accordance with institutional guidelines.

Cell fusion. Cell fusions of the splenocytes and the SP2/0 cells were carried out according to the standard fusion protocol (20). Fourteen days after fusion, the supernatants were harvested and screened for antibody production by ELISA. The selected hybridomas were grown in a CELLINE flask (INTEGRA Biosciences, Chur, Switzerland) and purified on a protein A column (Amersham Biosciences).

Screening by ELISA on mesothelin-Fc fusion protein. Screening of hybridomas was done by ELISA. Briefly, Nunc-Immuno plates (Nalge Nunc International, Rochester, NY) were coated with 2 μ g/mL of mesothelin-Fc protein. Plates were incubated overnight at 4°C. Then plates were blocked in blocking buffer (PBS with 25% DMEM, 5% FCS, 25 mmol/L HEPES, 0.5% bovine serum albumin, and 0.1% Azide) for 30 minutes at room temperature, washed twice with washing buffer (PBS with 0.05% Tween 20), and incubated with 100 μ L supernatant of hybridoma for 1 hour at room temperature. After washing, plates were incubated with a horseradish peroxidase-conjugated goat anti-mouse antibody diluted 1:2,000 in blocking buffer. Finally, plates were washed with washing buffer and 100 μ L immunopure tetramethylbenzidine substrate solution (Pierce) was added to each well. The color was allowed to develop for 2 to 5 minutes at room temperature and the reaction stopped by the addition of 50 μ L 2 N solution of sulfuric acid. The plates were read at OD 450 nm using an automated plate reader (Molecular Devices Corp., Sunnyvale, CA).

Anti-mesothelin MABs as control. MAb K1 was isolated and purified in our laboratory as previously described (2, 21). MAb 5B2 was obtained from Novocastra Laboratories, Ltd. (Newcastle upon Tyne, United Kingdom).

Antibody quantification. For the determination of MAB concentration, Sandwich ELISA was used. Nunc-Immuno plates (Nalge Nunc International) were coated with 2 μ g/mL of goat anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc., Grove, PA) in PBS overnight 4°C. One hundred microliters of blocking buffer was added to each well and then incubated for 30 minutes. After washing, serial diluted samples were added. As a standard immunoglobulin, mouse immunoglobulin classes and subclasses (Zymed Laboratories Inc., San Francisco, CA) were added. After washing, the bound MABs were detected by a 1-hour incubation with horseradish peroxidase-labeled goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) followed by tetramethylbenzidine substrate kit (Pierce). After adding 2 N-H₂SO₄ 50 μ L, the plates were read at OD₄₅₀. A standard curve was fit with the four-parameter logistic curve-fitting method.

Surface plasmon resonance assay. The affinity of the MABs to mesothelin-rFc was determined by surface plasmon resonance using BIAcore (Biacore, Piscataway, NJ). Three hundred and fifty response units of mesothelin-rFc was immobilized onto the biosensor chip, CM5 (using the BIAcore amine coupling kit). On and off rates of MABs were measured in PBS by injecting 25 μ g/mL of each MAB over the chip surface for 5 minutes and then allowing the bound material to dissociate for 5 minutes by flowing only the buffer solution over the chip. Binding kinetics were analyzed using BIAevaluation 2.1 software (22).

Flow cytometry. Cultured cells (2×10^5) were dissociated with dissociation buffer (Sigma-Aldrich, St. Louis, MO). Each sample was washed twice in FACS buffer (PBS plus 5% fetal bovine serum, and 0.1% sodium azide). One hundred microliters of each hybridoma supernatant was added to the cells and incubated for 1 hour at 4°C. Cells were then washed twice with FACS buffer, resuspended in 100 μ L of a secondary antibody (R-PE conjugated goat anti-mouse IgG diluted

1:100; Biosource, Camarillo, CA) and incubated for another hour at 4°C. Finally, cells were washed twice and analyzed on a FACSCalibur machine (Becton Dickinson, Franklin Drive, NJ) using the CellQuest software.

Isotyping. The isotype of selected MABs was determined using a mouse immunoglobulin isotyping kit (Roche Applied Science, Indianapolis, IN).

Western blot. Reactivity of anti-mesothelin MABs to SDS-denatured antigen was tested in a Western blot analysis. Mesothelin-Fc and CD25-Fc were separated on 4% to 20% SDS polyacrylamide gels (Bio-Rad, Hercules, CA) under reducing conditions. Proteins were transferred to a 0.2 µm Immobilon-P polyvinylidene difluoride membrane (Bio-Rad) in transfer buffer [25 mmol/L Tris-HCl, 192 mmol/L glycine, 30% (v/v) methanol (pH 8.3)] at 4°C for 1 hour at 240 mA. After blocking with blocking solution (Roche Applied Science), the membrane was incubated with 1 µg/mL of each MAB for 1 hour at room temperature. The bound MABs were detected with alkaline phosphatase-labeled goat anti-mouse IgG (Biosource) and BDIP/nitroblue tetrazolium substrate (Pierce).

Immunohistochemistry. Archival paraffin-embedded formaldehyde-fixed tissue sections from patients with mesothelioma were evaluated for mesothelin expression (15). The sections were deparaffinized in xylene, followed by graded ethanol hydration into water. The sections were treated for antigen-retrieval either using: (a) a commonly used commercial antigen unmasking solution (Vector, Burlingame, CA); (b) citraconic anhydride in a boiling water bath at 97°C for 60 minutes (23); or (c) treatment with 3 mol/L urea in a boiling water bath for 60 minutes. These treatments were followed by blocking with 1% bovine serum albumin in PBS for 30 minutes at 23°C, then incubation with primary antibodies in 1% bovine serum albumin-PBS for 60 minutes at room temperature. Primary mouse antibodies 5B2 (Novocastra Laboratories), MAB K1, MAB MB, or MAB MN were used at (or at a preparation dilution equivalent to) 10 µg/mL. After washing in PBS, the sections were incubated with affinity-purified goat anti-mouse IgG conjugated to horseradish peroxidase (Jackson ImmunoResearch) at 25 µg/mL in 1% bovine serum albumin-PBS for 30 minutes, followed by detection of peroxidase with diaminobenzidine-peroxide substrate solution for 10 minutes at 23°C. The sections were counterstained with hematoxylin.

Results

Generation of a recombinant mesothelin-Fc fusion protein and generation of MABs. To obtain MABs that recognize the

extracellular domain of mesothelin, we used a DNA immunization protocol followed by protein immunization for the final boost prior to cell fusion (24). The mesothelin-Fc protein used for immunization and screening was prepared from the medium of transfected HEK293T cells by purification on protein A-Sepharose (22). Six mesothelin-deficient mice were injected intradermally with DNA prepared from the plasmid, pcDNA3CAK1-9. Blood was collected from the mice after multiple injections and the antibody titer was determined by ELISA on plates coated with mesothelin-Fc protein (data not shown). The two mice with the highest titer ($>10^5$) were sacrificed. Spleen cells from each mouse were fused to myeloma cells following standard procedures (20). Supernatants from clones of hybridoma cells were screened using ELISA. To determine the specificity of the antibodies, the screening was done on plates coated with mesothelin-Fc and CD25-Fc. The latter is a negative control that excludes antibodies reacting with the Fc portion of the fusion. Finally, 17 hybridomas were identified that reacted selectively with mesothelin-Fc and not with CD25-Fc. The topographical epitopes of these MABs were identified based on the mutual competition of all possible pairs of the MABs (25). Two topographical epitope groups were identified. One group had 2 clones and contained MAB MB, whereas the other group had 15 clones and contained MAB MN, which competes with and belongs to the same epitope group as MAB K1. The characteristics of the two new MABs and the two standard anti-mesothelin MABs, K1 and 5B2, are shown in Table 1. The affinities of the new anti-mesothelin MABs determined by BIAcore (K_{ds} of 1.0 and 0.6 nmol/L) are much higher than previous MABs. Because the new MABs have high affinities for native mesothelin in the BIAcore format, we examined their performance in other types of assays. In these studies, we compared them with the two commercially available antibodies and found them to be useful in all the assays for mesothelin, whereas the two others did not detect mesothelin in all assay conditions. The data is shown below and summarized in Table 1.

Flow cytometry analysis of the anti-mesothelin supernatants. The 17 supernatants were tested by FACS for their

Table 1. Characterization of anti-mesothelin MABs

MABs	Isotype*	Affinity [†] (nmol/L)	FACS [‡]	ELISA [§] (ng/mL)	Western blot (ng)	Immunohistochemistry [¶]	Epitope**
MN	IgG _{2a}	1.0	3.5	1.1	2	+	1
MB	IgG _{2a}	0.6	3.3	1.9	2	+	2
K1	IgG ₁	12	2.5	1,000	25	+	1
5B2	IgG ₁	NA ^{††}	1.3	1,000	25	+	NA

*All MABs contained a κ light chain.

† Affinity to mesothelin-Fc in solution determined by BIAcore.

‡ Reactivity to H226 cells in FACS (log geometric mean of fluorescence intensity). Each MAB (1 µg/mL) was incubated with H226 (mesothelin-positive) cells and the bound MAB was detected by PE-labeled anti-mouse IgG. FACS histograms are shown in Fig 1. The values are geometric means of FACS signals. All the anti-mesothelin MABs reacted to H226 cells.

§ Reactivity to ELISA. ELISA plates were coated with mesothelin-Fc. After incubation with each MABs, the bound MAB was detected by HRP-labeled anti-mouse IgG. The values are the amounts of MABs that showed OD₄₅₀ = 0.5.

|| Reactivity to SDS-denatured mesothelin-Fc in Western blot (Fig. 3). The values are the minimum amount of mesothelin-Fc from which each MAB can be detected with.

¶ The results of K1 and 5B2 were cited from previous reports (2, 8, 9).

** Topological group of epitopes identified based on the mutual competition of the MABs (24).

†† Not applicable because 5B2 does not bind well to mesothelin-Fc.

ability to bind to the Panc 3.014 mesothelin-expressing cell line. All 17 antibodies bound to Panc 3.014 cells but did not bind to the A431 mesothelin negative cells (data not shown). One antibody from each epitope group, MN and MB, was selected for further study based on the highest signal on FACS. Representative FACS analysis of the new antibodies MN and MB, as well as MAb K1 and MAb 5B2 on H226 lung cancer cells, A1847 ovarian cancer cells, HeLa cervical cancer cells, and Panc 3.014 pancreatic cancer cells are shown in Fig. 1. The data in Fig. 1 show that MAbs MN and MB generated a large increase in fluorescence intensity compared with the cells incubated with the control anti-CD30 antibody. The signal was highest on H226 and Panc 3.014 cells followed by A1847 and HeLa cells. MAb K1 also reacted with H226, Panc 3.014, and A1847 cells but the signal was weaker than with MAbs MN or MB. Using MAb 5B2, the fluorescence signal was weak on H226 and negative on A1847, HeLa, and Panc 3.014 cells. With all antibodies, no reactivity was detectable in mesothelin-negative cells, A431 (data not shown). These data show that MAbs MN and MB are superior to the other MAbs for FACS analysis. The isotype of MAbs MN and MB are IgG_{2a}.

ELISA. To assess the reactivity of the MAbs in an ELISA format, plates coated with 2 µg/mL of mesothelin-Fc were exposed to increasing concentrations of each of the MAbs. Figure 2A shows representative data from triplicate experiments. MAb MN showed the strongest signal followed by MAb MB. MAbs K1 and 5B2 showed a very weak signal. In a control ELISA using plates coated with CD25-Fc, none of the antibodies showed a signal (data not shown). Thus, MAb MN seems to be the best choice for detection of mesothelin in this ELISA format. In another ELISA format, plates were coated with mesothelin produced in *E. coli* and the reactivity of the antibodies compared. Figure 2B shows representative data from triplicate experiments. MN showed a very strong signal followed by 5B2, which showed a weak signal; K1 and MB, showed a very weak signal.

Western blot. The reactivity of the anti-mesothelin MAbs to SDS-denatured antigen was tested in a Western blot analysis in which various amounts of mesothelin-Fc were loaded on a gel and blotted with each antibody. Figure 3 shows that MAbs MN

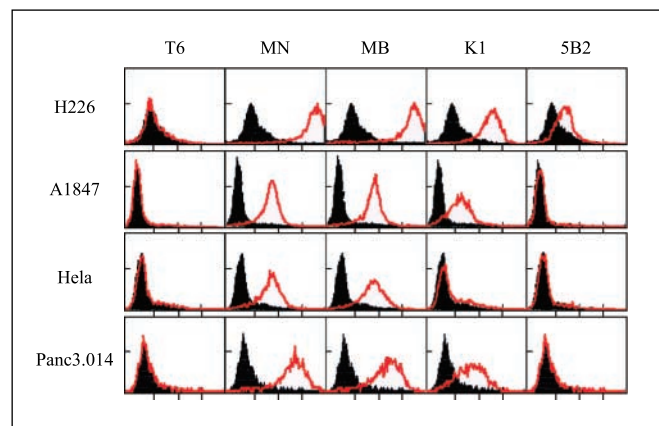


Fig. 1. FACS analysis of anti-mesothelin MAbs with different cells. Cells were incubated with the anti-mesothelin MAbs (1 µg/mL) or anti-CD30 MAbs, T6, as a negative control, followed by PE-labeled goat anti-mouse IgG. Each histogram shows the staining with first antibody (red line) and without first antibody (gray shadow). First antibody and cells used are shown. Mean fluorescence intensities for Table 1 were measured under the same conditions for all experiments.

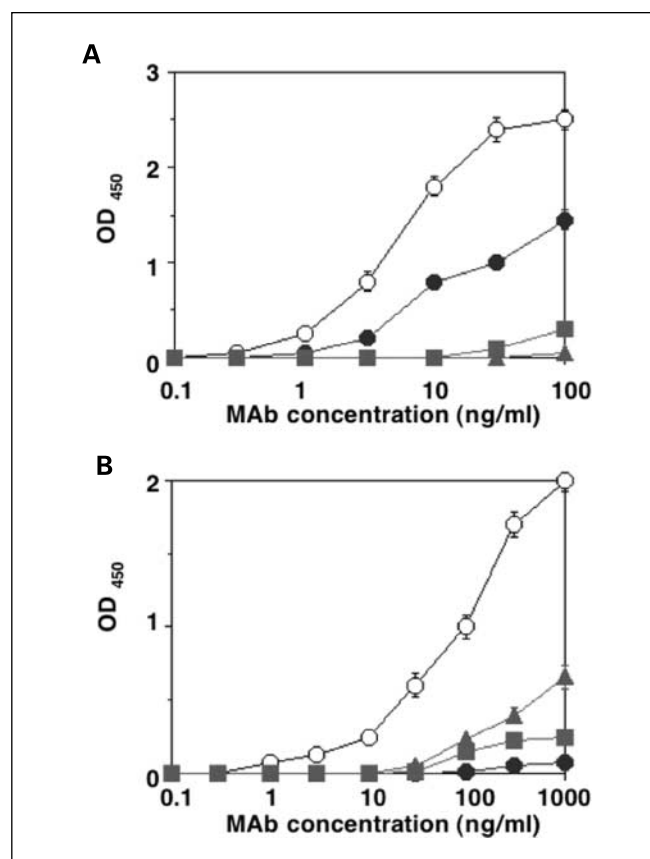


Fig. 2. MAb reactivity on ELISA coated with mesothelin-Fc (A), or bacterial maltose-binding protein - mesothelin (B). ELISA plates were coated with mesothelin-Fc (2 µg/mL) or bacterial mesothelin (2 µg/mL). Then, ELISA plates were incubated with anti-mesothelin MAbs, MN (○), MB (●), K1 (■), or 5B2 (▲), followed by horseradish peroxidase - conjugated goat anti-mouse IgG. Experiments were done in triplicate. Points, mean; bars, ± SD (n = 3).

and MB are 10 times more sensitive in detecting mesothelin on the Western blot than MAbs K1 and 5B2. For specificity controls, we showed that none of the antibodies reacted with the CD25-Fc control protein. In addition, monoclonal anti-CD30 antibodies did not react with mesothelin-Fc or CD25-Fc protein. This result suggests that these MAbs probably recognize a linear epitope on mesothelin.

Immunohistochemistry. The patterns seen using peroxidase immunohistochemistry with different anti-mesothelin antibodies in the same area of the same case of mesothelioma are shown in Fig. 4. In (A), sections treated with a commercial antigen retrieval reagent (Vector) show very poor reaction with a case of mesothelioma using MAb K1, whereas (A') shows the result following antigen retrieval using 3 mol/L urea. The levels of reactivities detected under these conditions are comparable to those seen in the detection of mesothelin using MAb K1 in frozen sections (data not shown). Thus, MAb K1 can detect mesothelin expression, but only in frozen sections or paraffin sections treated using aggressive antigen retrieval, and is much less effective using more common commercial antigen retrieval reagents. As described below, a new aggressive antigen retrieval method using citraconic anhydride also improved K1 reactivity. On the other hand, MAb 5B2, a commercial antibody from Novocastra Laboratories, shows good reactivity after commercial antigen retrieval (B), a level of intensity similar to MAb MN

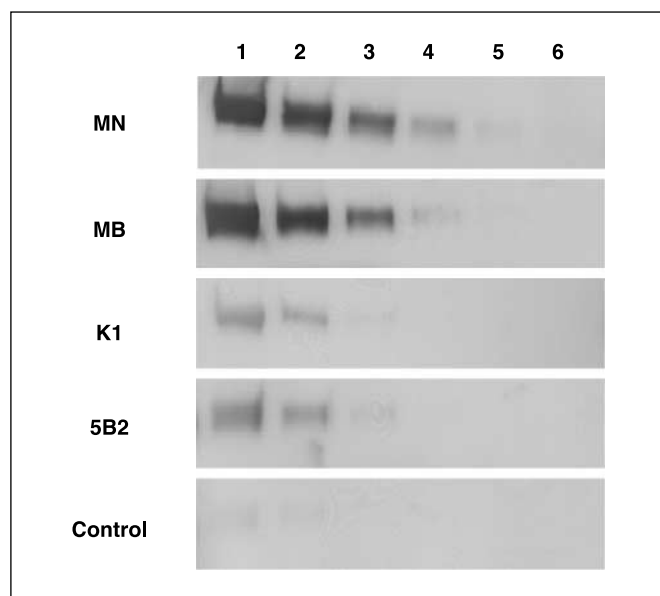


Fig. 3. Western blot analysis of anti-mesothelin MAbs. Mesothelin-Fc (lane 1, 100 ng; lane 2, 25 ng; lane 3, 6 ng; lane 4, 2 ng; and lane 5, 0.4 ng) and CD25-Fc (lane 6, 50 ng) were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were probed with each anti-mesothelin MAbs (1 μ g/mL) or anti-CD30 MAb, T6 (control), followed by ALP-goat anti-mouse IgG and BCIP/nitroblue tetrazolium substrate. Each experiment was done under the same conditions. Data are summarized in Table 1.

shown in (D). MAb MB, however, shows superior reactivity with commercial antigen retrieval, as shown in (C). This pattern was observed in three separate cases of mesothelioma examined using these reagents. This suggests that MAb MB may be a superior reagent for use with commonly used commercial immunohistochemical procedures in archival paraffin-embedded tissues. We also showed that our newly established MAbs are specific for mesothelin-expressing cells using tissue arrays with

different cancers and normal controls (Fig. 5; Table 2). These latter comparisons were made using citraconic anhydride antigen retrieval, a recently introduced reagent for antigen retrieval that is effective for epitopes that are difficult to unmask, including the K1-reactive epitope (ref. 23; data not shown).

Discussion

We have succeeded in producing useful antibodies to the membrane protein mesothelin by DNA immunizations followed by a single boost of a recombinant mesothelin-Fc fusion protein produced by human cells. The newly established MAbs react strongly and specifically to two different epitopes on the native form of the mesothelin as well as with denatured mesothelin. These data confirm previous results with other proteins that DNA immunization followed by boost with a recombinant protein is an easy and useful method to produce MAbs against cell surface antigens.

For immunization, we used mesothelin-deficient mice, because they should not be tolerant to epitopes on human mesothelin that are also present in mouse mesothelin and therefore should react to more epitopes on mesothelin than normal mice (5). Using a mutual competition assay, we evaluated the epitopes recognized by the 17 new clones as well as those recognized by MAb K1 and immunotoxin SS1P (data not shown). We found that we obtained antibodies against only two epitopes. One of these epitopes was recognized by the K1 antibody previously made in normal mice. MAbs MN, K1 and immunotoxin SS1P, in which the Fv was obtained from an antibody phage library are in the same epitope group. MAb K1 was generated by immunization with the human ovarian cancer cell line OVCAR-3 (1, 2). On the other hand, the Fv of SS1P was cloned from the splenic mRNA of mice using antibody phage display. The mice were immunized with an expression vector coding for mesothelin.

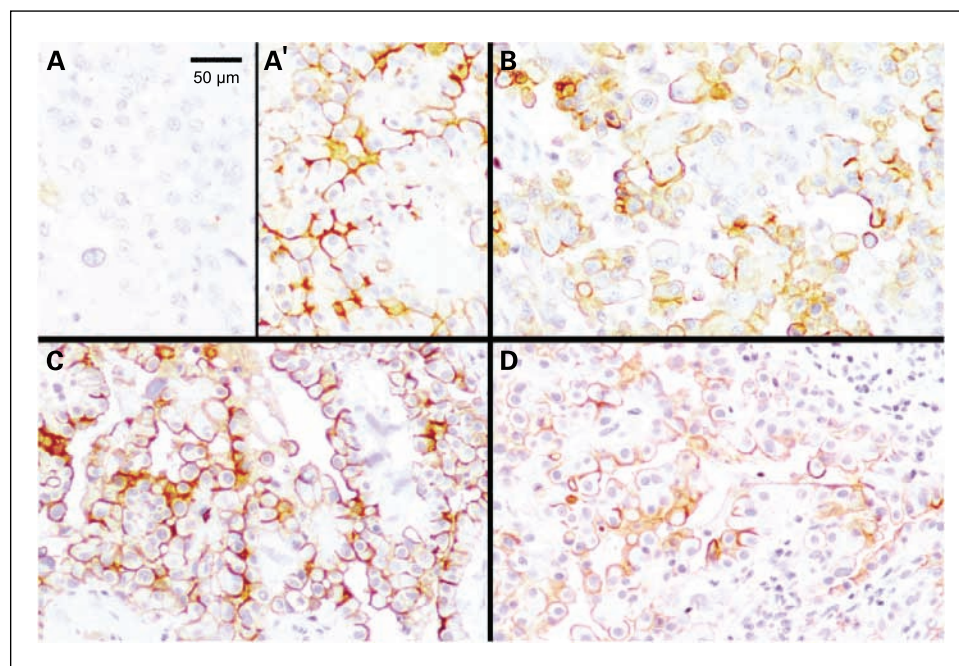
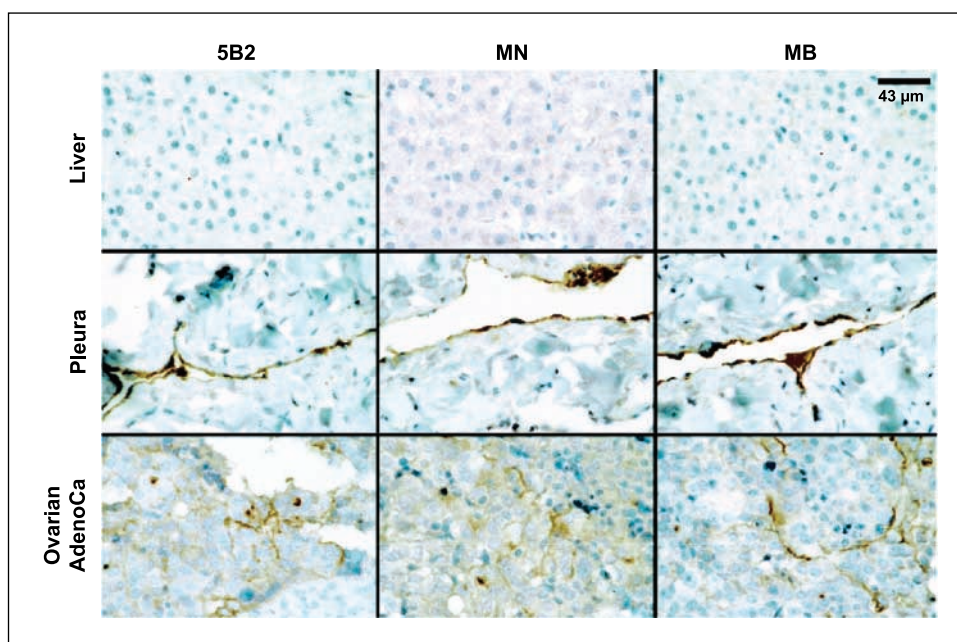


Fig. 4. Immunohistochemistry of anti-mesothelin MAbs. Peroxidase immunohistochemical results are shown from the same area of the same case of mesothelioma. Formaldehyde-fixed, paraffin-embedded tissue sections were treated with a commercial antigen retrieval procedure (Vector; A-D), but one section was treated with the 3 mol/L urea antigen retrieval (A'). Anti-mesothelin primary antibodies were used to label these sections as follows: A and A', MAb K1; B, MAb 5B2; C, MAb MB; and D, MAb MN. Note that MAb K1 labels poorly with commercial antigen retrieval, but is much stronger using the 3 mol/L urea treatment, whereas the other antibodies label well using commercial antigen retrieval, with MAb MB (C) showing the most intense reaction (magnification, $\times 200$; bar, 50 μ m).

Fig. 5. Immunohistochemistry of anti-mesothelin MABs on tissue arrays. Representative peroxidase immunohistochemical results are shown. Paraffin-embedded tissue sections (as shown in Table 2) were obtained from Biochain Institute (Hayward, CA) and treated with a citraconic anhydride antigen retrieval procedure (23). The complete results including other tissues are summarized in Table 2 (*bar*, 43 μ m).



Fifteen of the 17 newly established MABs reacted with this epitope. Because such antibodies are frequently generated by three different methods of immunization, this must be a dominant epitope (1, 11). MAB MN reacts with mesothelin-Fc protein and with mesothelin made in *E. coli*, showing that this epitope is present on recombinant mesothelin made in mammalian cells and in bacteria (Fig. 2). The epitope recognized by MAB MB does not react with mesothelin made in bacteria (Fig. 2B). MAB MB works very well for immunohistochemistry using a commercial antigen retrieval reagent (Vector) and should therefore be useful for such studies. On the other hand, even though it works for immunohistochemistry with commercial antigen retrieval methods, 5B2 reacts with the bacterial form of mesothelin, but not with the native mesothelin made in human cells.

The affinities of the new anti-mesothelin MABs were measured by BIAcore and found to have high affinities with K_d s of 1.0 and 0.6 nmol/L. These antibodies showed good performance when used for FACS analysis, ELISA, Western blot, and immunohistochemistry (Table 1). Thus, these MABs are useful for detecting the mesothelin protein using all types of immunologic assays. Several studies have described the usefulness of detecting tumor mesothelin expression by immunohistochemistry as an aid in the pathologic diagnosis of mesothelioma, ovarian cancer, and pancreatic cancer. Although initial studies using MAB K1 on frozen sections suggested that mesothelin could help distinguish between mesothelioma and adenocarcinoma, a recent study using MAB 5B2 noted diffuse mesothelin expression in some lung adenocarcinomas (1, 10). Because mesothelin immunostaining is a very sensitive marker for epithelial mesothelioma, a negative mesothelin immunostain suggests a diagnosis other than mesothelioma (9). Tumor mesothelin expression has also been shown to be useful for the diagnosis of ovarian cancer. A study that looked at mesothelin expression by a variety of different tumors showed strong mesothelin expression by nonmucinous ovarian cancers but not in a variety of tu-

mors with which these lesions may be confused; therefore, mesothelin staining can assist in establishing the differential diagnosis (10). Mesothelin expression in the majority of pancreatic ductal adenocarcinomas makes it a valuable tool

Table 2. Summary of immunohistochemical study on the tissue arrays

	5B2	MB	MN
Tumor			
Ovarian adenocarcinoma 1	+	+	+
Ovarian adenocarcinoma 2	focal+	focal+	focal+
Teratoma	—	—	—
Hepatoma			
Breast ductal adenocarcinoma	—	—	—
Leiomyoma	—	—	—
Squamous cell lung carcinoma	—	—	—
Astrocytoma	—	—	—
Renal cell carcinoma	—	—	—
Normal tissues			
Peritoneal mesothelium (on uterus)	+	+	+
Pleural mesothelium (on lung)	+	+	+
Brain	—	—	—
Breast	—	—	—
Kidney	—	—	—
Fallopian tube epithelial cells	—	—	—
Fallopian tube luminal fluid	+	+	+
Liver	—	—	—
Lung	—	—	—
Uterus (endometrium, myometrium)	—	—	—
Ovarian stroma 1	—	—	—
Ovarian stroma 2	—	—	—
Placenta	—	—	—

NOTE: +, stained; —, not stained.

for diagnosis of these tumors (3). In a study by Hornick et al., mesothelin in combination with other tumor markers was useful in distinguishing metastatic pancreatic adenocarcinoma from bile duct adenomas and hamartomas of the liver that are at times difficult to diagnose by light microscopy alone (26). Also, mesothelin immunostaining was shown to be valuable in distinguishing metastatic pancreatic mucinous adenocarcinomas in the ovaries from primary ovarian mucinous neoplasm; this is critical for proper patient management (27). Mesothelin staining can also help in the interpretation of pancreatic fine-needle aspiration given the cytologic overlap of neoplastic and reactive processes (28).

Hellstrom et al. have identified a soluble member of the mesothelin/MPF family, called soluble mesothelin-related protein that is elevated in the serum of the majority of patients with ovarian cancer (17). A recent study that evaluated serum CA 125 and soluble mesothelin-related protein in ovarian cancer patients and normal risk controls showed that using both markers together may be superior to CA 125 alone for ovarian cancer screening and diagnosis (29). In addition, soluble mesothelin-related protein has been shown to be a

useful marker for the diagnosis of pleural mesothelioma and to follow disease progression (18). We have evidence that a small amount of cell-bound mesothelin is shed into the serum; this could be a valuable test for the diagnosis and follow-up of patients with mesothelin-expressing cancers.³

The detection of mesothelin expression by immunohistochemistry, besides being useful to the pathologists in making a diagnosis, is also important clinically to select patients for therapies targeting mesothelin (8) and the development of assays to measure serum mesothelin would be useful for the diagnosis and follow-up of patients with mesothelin-expressing cancers.

In conclusion, we have established new MABs that react with mesothelin. Two of these, MN and MB, have better overall performance characteristics than previous MABs and should be useful in studying the biology of mesothelin and also aid in the diagnosis and therapy of mesothelin-expressing cancers.

³ Our unpublished data.

References

- Chang K, Pai LH, Pass H, et al. Monoclonal antibody K1 reacts with epithelial mesothelioma but not with lung adenocarcinoma. *Am J Surg Pathol* 1992;16:259–68.
- Chang K, Pastan I, Willingham MC. Isolation and characterization of a monoclonal antibody reactive with ovarian cancers and normal mesothelium. *Int J Cancer* 1992;50:373–81.
- Argani P, Iacobuzio-Danahue C, Ryu B, et al. Mesothelin is overexpressed in the vast majority of ductal adenocarcinomas of the pancreas: identification of a new pancreatic cancer marker by serial analysis of gene expression (SAGE). *Clin Cancer Res* 2001;7:3862–8.
- Chang K, Pastan I. Molecular cloning of mesothelin, a differentiation antigen present on mesothelium, mesothelioma, and ovarian cancers. *Proc Natl Acad Sci U S A* 1996;93:136–40.
- Bera TK, Pastan I. Mesothelin is not required for normal mouse development on reproduction. *Mol Cell Biol* 2000;20:2902–6.
- Rump A, Morikawa Y, Tanaka M, et al. Binding of ovarian cancer antigen CA125/MUC16 to mesothelin mediates cell adhesion. *J Biol Chem* 2004;279:9190–8.
- Hassan R, Kreitman RJ, Pastan I, Willingham MC. Localization of mesothelin in epithelial ovarian cancer. *Appl Immunohistochem Mol Morphol*. In press 2005.
- Hassan R, Bera T, Pastan I. Mesothelin: a new target for immunotherapy. *Clin Cancer Res* 2004;10:3937–42.
- Ordenez NG. Value of mesothelin immunostaining in the diagnosis of mesothelioma. *Mol Pathol* 2003;16:192–7.
- Ordenez NG. Application of mesothelin immunostaining in tumor diagnosis. *Am J Surg Pathol* 2003;27:1418–28.
- Kreitman RJ, Wilson WH, Bergeron K, et al. Efficacy of the anti-CD22 recombinant immunotoxin BL22 in chemotherapy-resistant hairy-cell leukemia. *N Engl J Med* 2001;345:241–7.
- Hassan R, Lerner MR, Benbrook D, et al. Antitumor activity of SS(dsFv)PE38 and SS1(dsFv)PE38, recombinant anti-mesothelin immunotoxins against human gynecologic cancers grown in organotypic cultures *in vitro*. *Clin Cancer Res* 2002;8:3520–6.
- Onda M, Nagata S, Tsutsumi Y, et al. Lowering the isoelectric point of the Fv portion of recombinant immunotoxins leads to decreased nonspecific animal toxicity without affecting antitumor activity. *Cancer Res* 2001;61:5070–7.
- Chowdhury PS, Viner JL, Beers R, Pastan I. Isolation of a high-affinity stable single-chain Fv specific for mesothelin from DNA-immunized mice by phage display and construction of a recombinant immunotoxin with anti-tumor activity. *Proc Natl Acad Sci U S A* 1998;95:669–74.
- Hassan R, Kreitman RJ, Strauss L, et al. SS1(dsFv)PE38 anti-mesothelin immunotoxin in advanced malignancies: phase I and pharmacokinetic study of alternative-day infusion. *Proc Am Soc Clin Oncol* 2002;21:29a.
- Thomas AM, Santarsiero LM, Lutz ER, et al. Mesothelin-specific CD8+ T cell response provide evidence of *in vivo* cross-priming by antigen-presenting cells in vaccinated pancreatic cancer patients. *J Exp Med* 2004;200:297–306.
- Scholler N, Fu N, Yang Y, et al. Soluble member(s) of the mesothelin megakaryocyte potentiating factor family are detectable in sera from patients with ovarian carcinoma. *Proc Natl Acad Sci U S A* 1999;96:11531–6.
- Robinson BWS, Creaney J, Lake R, et al. Mesothelin-family protein and diagnosis of mesothelioma. *Lancet* 2003;362:1612–6.
- Bernstein KE, Alexander CB, Mage RG. Nucleotide sequence of a rabbit IgG heavy chain from the recombinant F-I haplotype. *Immunogenetics* 1983;18:387–97.
- Nelson PN, Reynolds GM, Waldron EE, Ward E, Giannopoulos K, Murray PG. Monoclonal antibodies. *Mol Pathol* 2000;53:111–7.
- Hassan R, Wu C, Brechbiel MW, Margulies I, Kreitman RJ, Pastan I. 111-Indium-labelled monoclonal antibody K1: biodistribution study in nude mice bearing a human carcinoma xenograft expressing mesothelin. *Int J Cancer* 1999;80:559–63.
- Nagata S, Onda M, Numata Y, et al. Novel anti-CD30 recombinant immunotoxins containing disulfide-stabilized Fv fragments. *Clin Cancer Res* 2002;8:2345–55.
- Namimatsu S, Ghazizadeh M, Sugisaki Y. Reversing the effect of formalin fixation with citraconic anhydride and heat: a universal antigen retrieval method. *J Histochem Cytochem* 2005;53:3–11.
- Chowdhury PS, Pastan I. Analysis of cloned Fvs from a phage display library indicates that DNA immunization mimic antibody response generated by cell immunizations. *J Immunol Methods* 1999;231:83–91.
- Nagata S, Numata Y, Onda M, et al. Rapid grouping of monoclonal antibodies based on their topographical epitopes by a label-free competitive immunoassay. *J Immunol Methods* 2004;292:141–55.
- Hornick JL, Lauwers GY, Odze RD. Immunohistochemistry can help distinguish metastatic pancreatic adenocarcinomas from bile duct adenomas and hamartomas of the liver. *Am J Surg Pathol* 2005;29:381–9.
- Cao D, Ji H, Ronnett BM. Expression of mesothelin, fascin, and prostate stem cell antigen in primary ovarian mucinous tumors and their utility in differentiating primary ovarian mucinous tumors from metastatic pancreatic mucinous carcinomas in the ovary. *Int J Gynecol Pathol* 2005;24:67–72.
- McCarthy DM, Maitra A, Argani P, et al. Novel markers of pancreatic adenocarcinoma in fine-needle aspiration: mesothelin and prostate stem cell antigen labeling increases accuracy in cytologically borderline cases. *Appl Immunohistochem Mol Morphol* 2003;11:238–43.
- McIntosh MW, Drescher C, Karlan B, et al. Combining CA125 and SMR serum markers for diagnosis and early detection of ovarian carcinoma. *Gynecol Oncol* 2004;95:9–15.

Clinical Cancer Research

New Monoclonal Antibodies to Mesothelin Useful for Immunohistochemistry, Fluorescence-Activated Cell Sorting, Western Blotting, and ELISA

Masanori Onda, Mark Willingham, Satoshi Nagata, et al.

Clin Cancer Res 2005;11:5840-5846.

Updated version Access the most recent version of this article at:
<http://clincancerres.aacrjournals.org/content/11/16/5840>

Cited Articles This article cites by 27 articles, 13 of which you can access for free at:
<http://clincancerres.aacrjournals.org/content/11/16/5840.full.html#ref-list-1>

Citing articles This article has been cited by 16 HighWire-hosted articles. Access the articles at:
<http://clincancerres.aacrjournals.org/content/11/16/5840.full.html#related-urls>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.

Preclinical evaluation of MORAb-009, a chimeric antibody targeting tumor-associated mesothelin

Raffit Hassan^{1*}, Wolfgang Ebel^{2*}, Eric L. Routhier², Rina Patel², J. Bradford Kline², Jingli Zhang¹, Qimin Chao², Sara Jacob², Howard Turchin², Lester Gibbs², Martin D. Phillips², Shiyama Mudali³, Christine Iacobuzio-Donahue³, Elizabeth M. Jaffee⁴, Maria Moreno⁵, Ira Pastan¹, Philip M. Sass², Nicholas C. Nicolaides² and Luigi Grasso²

¹Laboratory of Molecular Biology, National Cancer Institute, Bethesda, MD, USA

²Morphotek Inc., Exton, PA, USA

³Drug Development Program, The Sol Goldman Pancreatic Cancer Research Center, Johns Hopkins University, Baltimore, MD, USA

⁴Department of Oncology, The Sol Goldman Pancreatic Cancer Research Center, Johns Hopkins University, Baltimore, MD, USA

⁵Obstetrics and Gynecology, VU Medical Centre, Amsterdam, The Netherlands

*These authors contributed equally to this work

Communicated by: V Cerundolo

Novel therapeutic agents that are safe and effective are needed for the treatment of pancreatic, ovarian, lung adenocarcinomas and mesotheliomas. Mesothelin is a glycosyl-phosphatidyl inositol (GPI)-linked membrane protein of 40 kDa over-expressed in all pancreatic adenocarcinoma and mesothelioma, in >70% of ovarian adenocarcinoma, and in non-small cell lung and colorectal cancers. The biological functions of mesothelin are not known, although it appears to be involved in cell adhesion via its interaction with MUC16. We have recently developed MORAb-009, a mouse-human chimeric IgG1κ monoclonal antibody with an affinity of 1.5 nM for human mesothelin. Here we provide evidence that MORAb-009 prevents adhesion of mesothelin-bearing tumor cells to MUC16 positive cells and can elicit cell-mediated cytotoxicity on mesothelin-bearing tumor cells. Treatment that included MORAb-009 in combination with chemotherapy led to a marked reduction in tumor growth of mesothelin-expressing tumors in nude mice compared to chemotherapy or MORAb-009 treatment alone. No adverse effects of MORAb-009 were noted during toxicology studies conducted in non-human primates. The preclinical data obtained from our studies warrants pursuing clinical testing of MORAb-009. We have in fact initiated a Phase I clinical study enrolling patients with mesothelin-positive pancreatic, mesothelioma, non-small cell lung and ovarian cancers.

Keywords: preclinical drug evaluation, monoclonal antibody, mesothelin, antibody-dependent cellular cytotoxicity

Introduction

Mesothelin is a glycosyl-phosphatidyl inositol-linked membrane glycoprotein expressed in a variety of cancers (1, 2). Immunohistochemistry studies have shown that mesothelin is over-expressed in virtually all pancreatic ductal adenocarcinomas and mesotheliomas (3-5), a high percentage of epithelial ovarian cancers (including fallopian tube and primary peritoneal malignancies), as well as non-small cell carcinomas of the lung (6-10). Expression in normal tissues is limited to mesothelial cells lining the pleura, pericardium and peritoneum (6).

Since mesothelin has recently been reported to bind to CA125/MUC16, one possible biological role of this membrane protein

may be linked to heterotypic cell adhesion, which may facilitate the metastatic spread of mesothelin-bearing cancer cells (11). Notwithstanding, the biologic functions of mesothelin remain to be fully defined. Analysis of the mesothelin amino acid sequence yielded no strong homologies to known protein functional domains, other than a C-terminal GPI-anchor motif (2). Moreover, mesothelin does not appear to be required for either normal development or reproduction since there were no apparent abnormalities in mesothelin knockout mice (12).

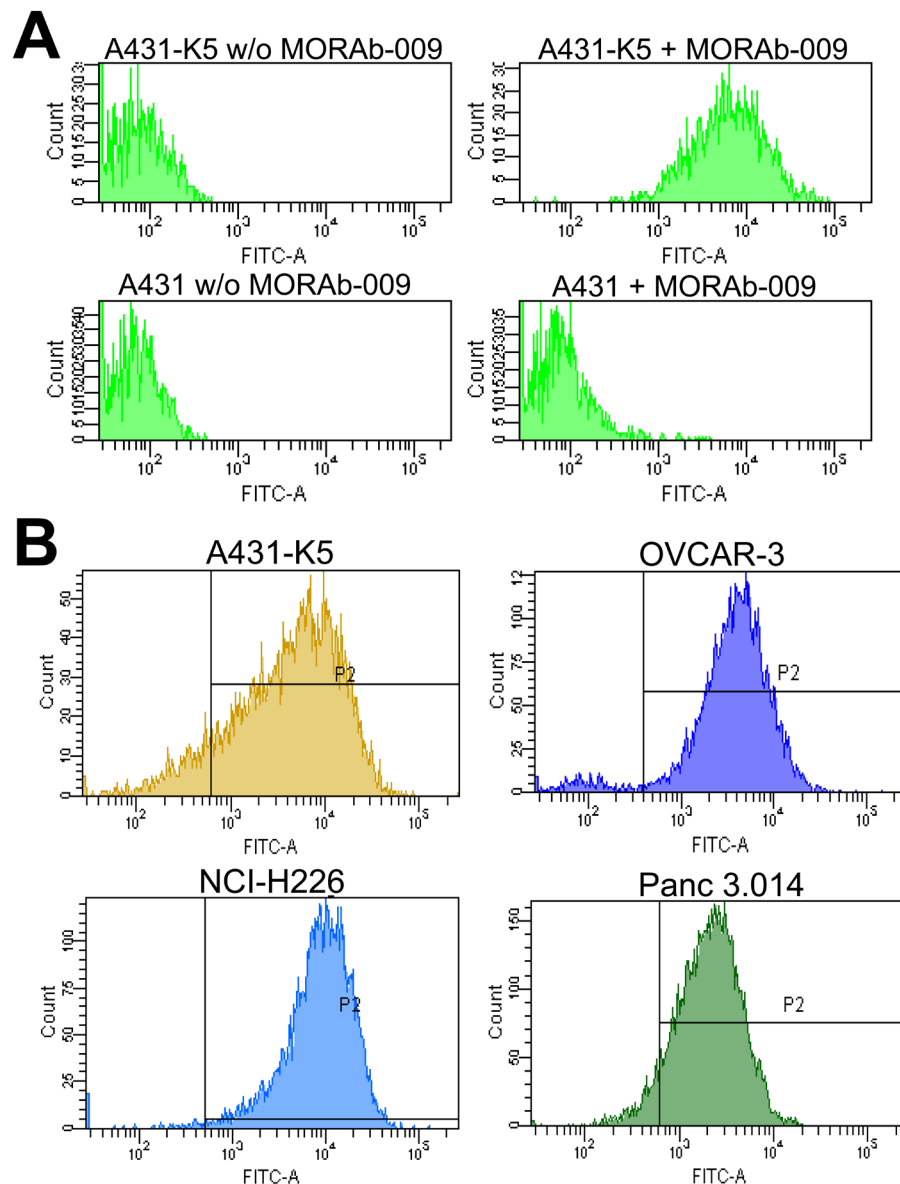
Two lines of clinical evidence suggest that mesothelin may be an important target for the treatment of mesothelin-bearing tumors. First, tumor responses were observed in a Phase I trial of the anti-mesothelin immunotoxin, SS1P, which included patients with mesotheliomas, ovarian and pancreatic cancer (13). In a different clinical trial, induction of cytotoxic T cell responses to mesothelin epitopes were exclusively obtained in three patients vaccinated with autologous mesothelin-positive tumor cells who had shown delayed-type hypersensitivity after vaccination (14).

Novel therapeutic agents that are safe and effective are needed for the treatment of pancreatic, ovarian, mesothelioma, and lung cancers. Over the years, monoclonal antibodies have proven to be well tolerated, disease antigen-specific, and able to deliver clinical benefits to patients with conditions that include chronic inflammation, lymphomas, and metastatic solid tumors (15). Here we report the preclinical evaluation of MORAb-009, a chimeric monoclonal antibody antagonizing human mesothelin to determine its suitability as an anti-tumor agent. MORAb-009 was able to mediate biological responses *in vitro*, including inhibition of mesothelin-dependent cell adhesion, antibody-dependent cellular cytotoxicity (ADCC), as well as the reduction of tumor growth in mice implanted with human cancer cells, while exhibiting no adverse effects in non-human primates.

Results

Generation of MORAb-009

The mouse antibody precursor of MORAb-009 was first isolated by panning a phage display library made from splenic mRNA of a mouse immunized with mesothelin cDNA on mesothelin-positive cells (16). The affinity of this mouse single-

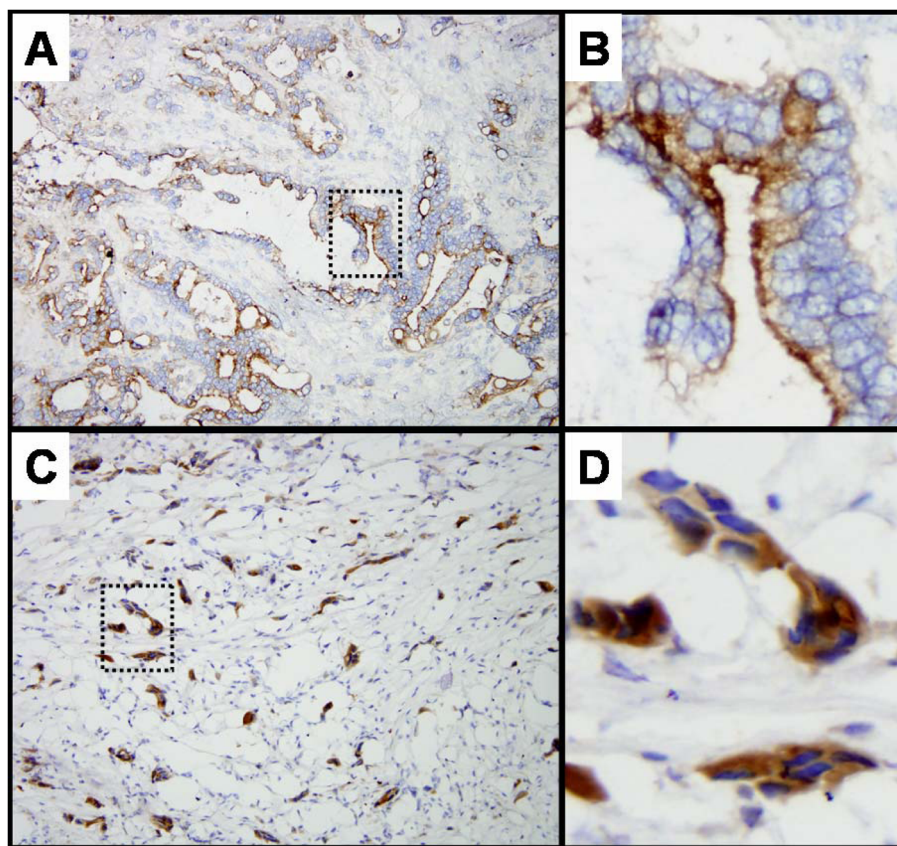
Figure 1

FACS analyses of tumor cells stained with MORAb-009. (A) Parental A431 cells, as well as cells expressing mesothelin (A431-K5), were reacted with FITC-labeled anti-human IgG antibody, with or without MORAb-009. Positive staining was observed only in the presence of MORAb-009 in A431-K5 cells, demonstrating specificity of the immune staining and lack of reactivity of MORAb-009 with other unrelated membrane proteins. (B) Tumor cells were reacted with FITC-labeled anti-human IgG antibody alone to determine background staining (not shown) and to create gate P2 to capture fluorescent signal above background. MORAb-009 was able to bind mesothelin expressed by ovarian (OVCAR-3), mesothelioma (NCI-H226), and pancreatic (Panc 3.014) cancer cells.

chain anti-mesothelin antibody was further optimized by re-engineering its variable regions to derive SS1(scFv) (17). Subsequently, we have cloned the heavy and light chain variable regions of SS1(scFv) and grafted them in frame with human IgG1 and kappa constant regions, respectively, yielding the genes coding for a chimeric (mouse/human) antibody designated MORAb-009 showing an affinity (K_D) of 1.5 nM. When aligned to its closest human germline immunoglobulin match, MORAb-009 exhibits a high amino acid sequence identity of 82.6% (excluding the complementarity determining regions), compared to a typical 90% identity of a humanized antibody.

MORAb-009 binds to cell surface mesothelin expressed by tumor cell lines

To test the binding specificity of MORAb-009, we conducted FACS analyses using the mesothelin negative and positive cells, A431 and A431-K5, respectively. A431-K5 cells were derived from human epidermoid carcinoma cells (A431) ectopically expressing membrane-bound human mesothelin (6). Surface binding by MORAb-009 was only observed on A431-K5 cells (Figure 1A, top right panel), indicating that MORAb-009 specifically bound mesothelin but not other membrane proteins expressed by these cancer cells. Mesothelin is frequently

Figure 2

Immunohistochemical staining of human pancreatic tumor tissues. Frozen sections of ductal pancreatic adenocarcinomas (A and C) were stained with MORAb-009. Membranous staining at the luminal and apical surface of neoplastic cells was detected. (B and D) Five-fold enlargement of dotted areas in A and C. Shown are clusters of neoplastic cells where approximately 75% of the cells stained positive with MORAb-009.

expressed in pancreatic, mesothelioma and ovarian cancers. We therefore tested whether MORAb-009 could recognize mesothelin on the cell surface of ovarian (OVCAR-3), mesothelioma (NCI-H226), and pancreatic (Panc 3.014) cancer cell lines known to express mesothelin. Significant MORAb-009 binding could be detected in all cell lines tested (Figure 1B) suggesting that this antibody is suitable for targeting tumor-associated mesothelin.

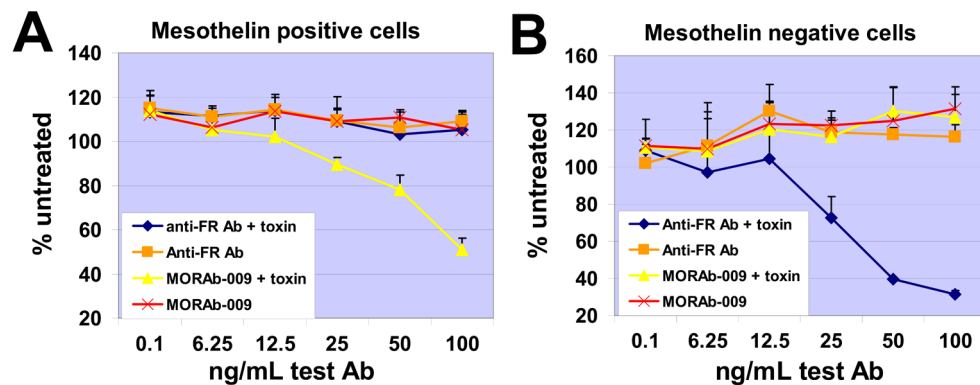
MORAb-009 targets tumor-associated mesothelin

Immunohistochemistry studies supported the ability of MORAb-009 to target mesothelin protein expressed in human pancreatic tumors. We detected membranous staining at the luminal and apical surface of neoplastic cells, although some cytoplasmic staining was also observed (Figure 2). Of the ten frozen pancreatic ductal adenocarcinomas (primary and metastatic disease) studied, all were found to be positive for MORAb-009 staining, with 50% of pancreatic cancer specimens showing strong positive labeling (3+) of at least 75% of the neoplastic cells and the remaining specimens exhibiting a less intensive staining (data not shown). This frequency of mesothelin staining seen with MORAb-009 in pancreatic cancer specimens matched that we have observed using the murine 5B2 antibody (data not shown) and is in line with previous immunohistochemistry studies using the same murine antibody (3). No relationship between tumor stage (primary versus

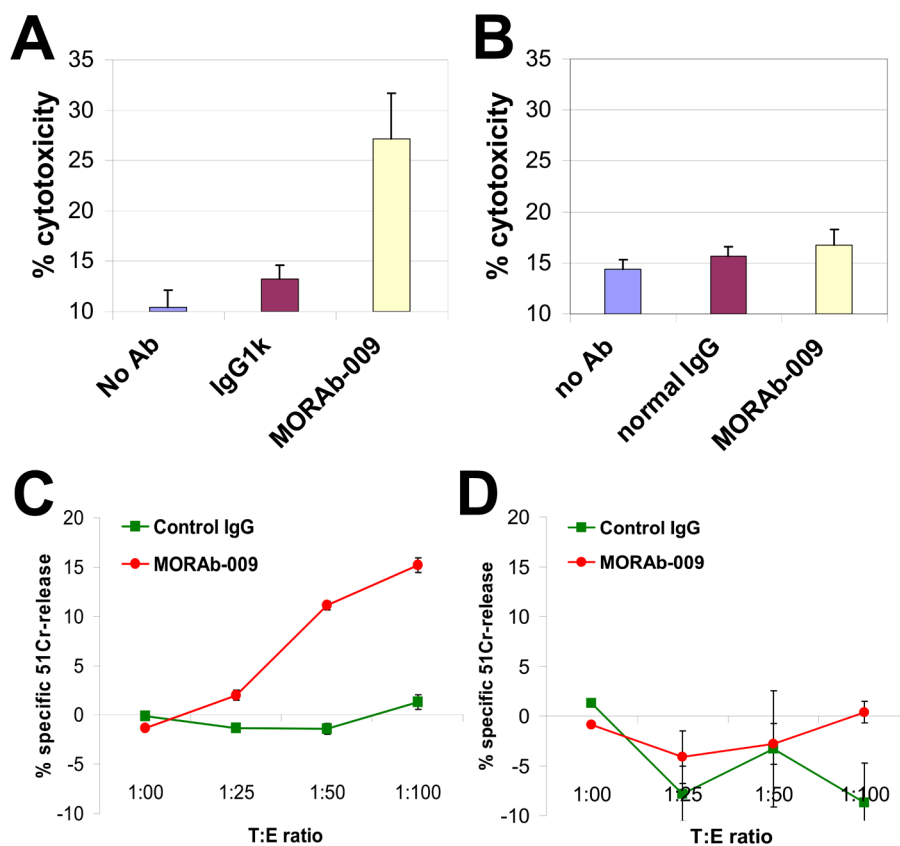
metastasis) and staining intensity was found, although we observed that well differentiated carcinomas tended to stain more robustly than poorly differentiated carcinomas.

Internalization of MORAb-009 upon binding to cell surface mesothelin

Saporin is a toxin that inhibits protein translation causing cell death only if it is internalized by cells and can be used to determine receptor internalization (18). To address whether MORAb-009 internalizes after binding cell surface mesothelin, we reacted MORAb-009 with an anti-human IgG conjugated with saporin, treated NCI-H226 cells (mesothelin positive) or IGROV-1 cells (mesothelin negative) with this complex, and measured cell death. The MORAb-009-saporin complex, but not MORAb-009 alone, caused significant death of NCI-H226 cells (Figure 3A). A control antibody specific to folate receptor α (FR α), which is not expressed in NCI-H226 cells, expectedly had no effect on cell viability whether alone or complexed with saporin-conjugated anti-human IgG. Neither MORAb-009-saporin complex nor MORAb-009 alone caused significant death of mesothelin negative, FR α positive IGROV-1 cells (Figure 3B). These results suggest that MORAb-009 can internalize after binding mesothelin on the cell surface.

Figure 3

Internalization of MORAb-009-mesothelin complex. (A) Mesothelin positive, FR α -negative cells were reacted with MORAb-009 alone, MORAb-009 complexed with saporin-conjugated anti-human mAb (toxin), anti-FR α mAb alone, or anti-FR α mAb complexed with toxin. Cell killing, and thus internalization, of MORAb-009-mesothelin complex was only seen with MORAb-009 complexed with toxin. (B) Same as in A using mesothelin-negative, FR α -positive cells. Internalization was only seen with anti-FR α mAb complexed with toxin.

Figure 4

MORAb-009 induces ADCC. (A) OVCAR-3 cells were used as target cells and reacted with 10 μ g/mL of MORAb-009 or control IgG in the presence of human PBMCs at a ratio of 1:25. ADCC activity was measured by LDH release and only noted with MORAb-009 treatment. (B) Same as in A, whereby target cells (Panc 3.27) express low or no mesothelin. (C) NCI-H226 cells were used as target cells and reacted with 10 μ g/mL of MORAb-009 or control IgG in the presence of human PBMCs at different ratios. ADCC activity was measured by 51 Cr release and only noted with MORAb-009 treatment. (D) Same as in C, whereby target cells (Panc 10.05) express no or low levels of mesothelin.

MORAb-009 can exert antibody-dependent cellular cytotoxicity

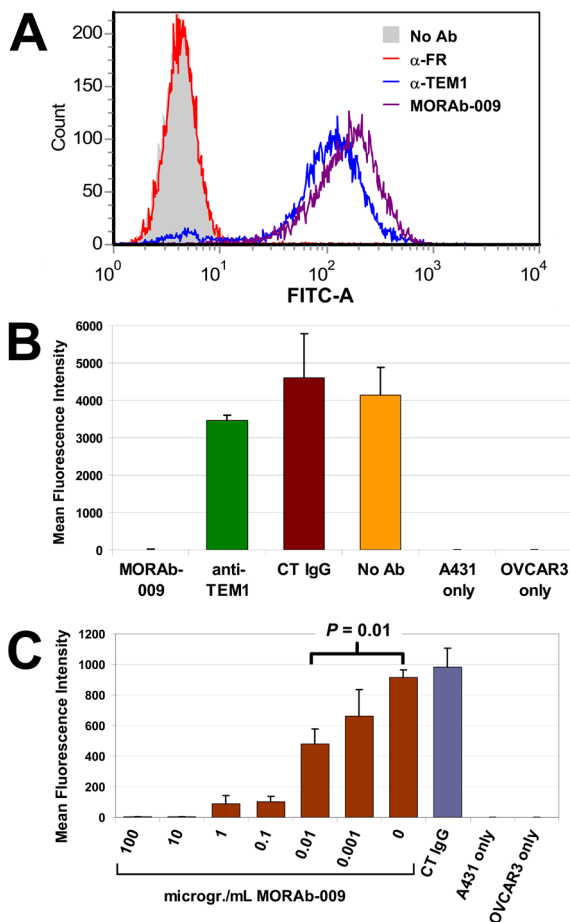
Antibody opsonization of tumor cells followed by the recruitment of immune killer cells eliciting cell-mediated cytotoxicity (ADCC) is thought to be an important mechanism of tumor cell cytotoxicity *in vivo*. To assess the ability of MORAb-009 to elicit this effector function, we used human cancer cell lines as targets for ADCC. In one strategy, measurement of lactate dehydrogenase (LDH) released by lysed cells was used to determine the extent of tumor cell killing using a constant target to effector ratio of 1:25. MORAb-009 exerted significant ADCC activity against OVCAR-3 cells (Figure 4A) but not on mesothelin negative pancreatic cancer cells, Panc 3.27 (Figure 4B). MORAb-009 also elicited significant ADCC activity on pancreatic cancer cells, Panc 3.014 (data not shown). We also used ^{51}Cr release to measure cell cytotoxicity and tested various target to effector ratios. MORAb-009 again mediated ADCC activity on mesothelin-expressing mesothelioma cells NCI-H226 (Figure 4C) but had no effect on human pancreatic cancer cells, Panc 10.05 (Figure 4D), which express low or undetectable mesothelin levels (data not shown). Maximum effect was seen at a target to effector ratio of 1:100. We did not measure significant complement-mediated cytotoxicity (CDC) by MORAb-009 (data not shown). Since our preliminary mapping identified an epitope for MORAb-009 at the amino terminus of mesothelin, one untested hypothesis is that mesothelin-bound MORAb-009 may be too far from the cell surface for the complement membrane attack complex to be effective.

CA125/MUC16 and mesothelin-dependent cell adhesion is blocked by MORAb-009

CA125/MUC16 has been identified as a ligand for mesothelin (11). CA125/MUC16 and mesothelin are both expressed in the peritoneal mesothelium as well as in some ovarian cancer cells and are believed to facilitate metastasis of cancer cells to the peritoneum by promoting cell attachment (19). In our preliminary studies, A431-K5 cells, but not parental A431 cells, could bind to CA125/MUC16 positive cells, demonstrating that this heterotypic cell interaction depends on mesothelin expression (data not shown). To explore the possibility that MORAb-009 interferes with mesothelin-CA125/MUC16 interaction, we established A431 cells (A431-K5-TEM) that expressed mesothelin as well as another surface receptor, endosialin/TEM1, but are negative for FR α . Figure 5A shows FACS analysis of A431-K5-TEM cells that exhibited positive staining after being reacted with either MORAb-009 or an anti-endosialin/TEM1 antibody. Expectedly, A431-K5-TEM cells did not stain with an anti-FR α antibody, demonstrating specificity of binding. We then measured adhesion of fluorescently labeled A431-K5-TEM cells onto CA125/MUC16 positive OVCAR-3 cells. Figure 5B outlines the results of this study. In the absence of OVCAR-3 cells, no adhesion of A431-K5-TEM cells to the uncoated surface of the microplate could be observed. Significant heterotypic cell binding was seen on a monolayer of OVCAR-3 cells in the absence of antibody treatment or with control IgG. In contrast, MORAb-009 completely abolished A431-K5-TEM cell adhesion onto OVCAR-3 cells. Although anti-endosialin/TEM1 antibody could bind A431-K5-TEM cells as strongly as MORAb-009 (Figure 5A), it did not inhibit cell-cell interaction, suggesting that MORAb-009-mediated inhibition of cell adhesion was due to specific disruption of mesothelin-CA125/MUC16 interaction rather than steric hindrance at the cell-cell interface. The ED $_{90}$ of this activity was

estimated around 100 ng/mL of MORAb-009 and a statistically significant inhibition was detected with concentrations as low as 10 ng/mL (Figure 5C).

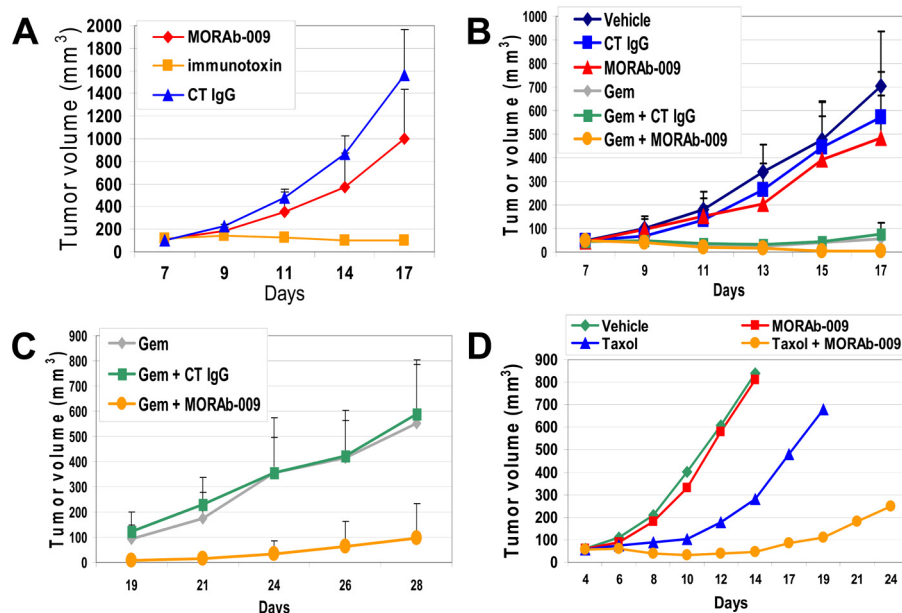
Figure 5



MORAb-009 blocks CA125/MUC16-mesothelin interaction. (A) FACS analysis of A431-K5-TEM cells reacted with no mAb, anti-FR α mAb, anti-TEM1 mAb, or MORAb-009. Equivalent positive staining was associated with the expression of cognate proteins (TEM-1 and mesothelin) but not with FR α , which is not expressed in these cells. (B) Fluorescently-labeled A431-K5-TEM cells were reacted with no mAb, anti-FR α mAb (CT IgG), anti-TEM1 mAb, or MORAb-009, and seeded on a monolayer of CA125/MUC16 positive OVCAR-3 cells (first four bars). Cell adhesion was completely blocked by MORAb-009 compared to the other treatments. (C) Same as in B, whereby MORAb-009 concentration ranged from 1 ng/mL to 100 μ g/mL. Blockade of CA125/MUC16-mesothelin interaction was seen with concentrations as low as 10 ng/mL.

MORAb-009 enhances the anti-tumor effects of chemotherapy *in vivo*

The *in vivo* anti-tumor effect of MORAb-009 in combination with chemotherapy was evaluated in immunodeficient mice bearing A431-K5 tumor xenografts. The number of receptor sites in these cells is comparable to that of other tumor cells endogenously expressing mesothelin (Figure 1B) and their implantation in mice consistently results in aggressive tumor

Figure 6

Effect of MORAb-009 on tumor growth. (A) A431-K5 cells were inoculated in the flank of nude mice to establish tumors of approximately 50 mm³ in size. On day 7, mice were treated with the control IgG1 Rituximab (CT IgG, 50 mg/kg), MORAb-009 (50 mg/kg), or mesothelin-specific immunotoxin SS1(scFv) (immunotoxin, 0.2 mg/kg). Average tumor size for each treatment group was calculated on day 7-17. (B and C) A431-K5 cells were inoculated as described in A. On day 7, mice were treated with vehicle, control IgG (CT IgG, 50 mg/kg), MORAb-009 (50 mg/kg), gemcitabine (Gem, 80 mg/kg), or combinations of these drugs (see Material and methods for regimens). Average tumor size for each treatment group was calculated on day 7-17 (panel B) and day 19-28 (panel C). Best anti-tumor responses were observed with gemcitabine plus MORAb-009. (D) Same model as in panels A-C, whereby mice were treated with vehicle, MORAb-009 (50 mg/kg), Taxol® (50 mg/kg), or combinations of these drugs. MORAb-009 enhanced the anti-tumor effect of Taxol®.

growth when compared to other mesothelin-positive cells. Preliminary studies using the A431-K5 tumor xenograft model showed moderate but statistically significant ($P = 0.01$) anti-tumor activity of MORAb-009 alone compared to the isotype control Rituximab, an IgG1 monoclonal antibody that targets the CD20 antigen not expressed on A431-K5 cells (Figure 6A). In this model, the mesothelin-specific immunotoxin SS1(scFv) could completely inhibit tumor growth. In subsequent studies, athymic nude mice bearing A431-K5 tumors were treated with MORAb-009 alone, gemcitabine alone (at a dose that can delay tumor growth without causing regression) or with the combination of the two agents. Seventeen days after inoculation of tumor cells, the average tumor size in mice treated with MORAb-009 alone was reduced compared to vehicle control and Rituximab alone treated mice, albeit this response was moderate and did not reach statistical significance ($P = 0.071$, Figure 6B). We observed significant tumor growth inhibition in mice treated with gemcitabine alone or in combination with MORAb-009 ($P < 0.001$), compared to control IgG (Rituximab) or MORAb-009 alone groups. Due to their tumor burden, animals in the vehicle control, Rituximab, and MORAb-009 single agent groups were sacrificed around day 17-18. The last dose of MORAb-009 or control IgG was administered on day 17, while we continued monitoring tumor volumes in the remaining groups for an additional 11 days (Figure 6C). Whereas tumors resumed vigorous growth in mice treated with gemcitabine alone, reaching an average volume of 600 mm³ by day 28, the average tumor volume in mice that also received MORAb-009 remained significantly smaller than 100 mm³ ($P = 0.001$, Figure 6C). Importantly, transient tumor remissions (tumor volumes 0-8 mm³) were only noted in the gemcitabine/

MORAb-009 treatment group (6 of the 10 mice) compared to none in the other groups, with two mice remaining tumor-free for the entire course of the study (35 days). Expectedly, the control IgG (Rituximab) had no effect on tumor growth whether administered alone or in combination with gemcitabine ($P = 0.548$). Since Taxol® is frequently used in the clinical setting as the first line therapy of mesothelin-expressing ovarian and lung adenocarcinomas, we also evaluated possible synergistic anti-tumor activity of MORAb-009 in combination with Taxol® using the above A431-K5 tumor xenograft model. As shown in Figure 6D, while treatment with MORAb-009 alone showed little tumor volume reduction and treatment with Taxol® alone only delayed tumor growth, we observed a more robust anti-tumor effect when Taxol® and MORAb-009 were used in combination. Importantly, four of the seven mice in the Taxol®/MORAb-009 combination treatment group exhibited complete tumor regression compared to none in the other groups.

MORAb-009 safety profile

Western blot analysis utilizing mesothelin-expressing tissues from rat, mouse and cynomolgus monkeys indicated lack of cross-reactivity of MORAb-009 to rodent species but significant binding to monkey tissues (data not shown). Immunohistochemistry (IHC) analysis confirmed similar staining pattern in normal tissues from human and cynomolgus macaque, with staining observed only in mesothelia. Therefore, a 23-day toxicology study with MORAb-009 was conducted in cynomolgus monkeys. This study employed relatively high doses of MORAb-009 administered over a short period to establish an initial safety profile of MORAb-009. There were no

Table 1

Mean serum pharmacokinetic parameters of MORAb-009 in male and female monkeys following intravenous infusion.

Dose (mg/kg/occasion)	Total Dose (mg/kg)	Gender	AUC _{0-529.5h} (μg.h/mL)	C _{max} (μg/mL)	T _{max} (h)
2	14	Female	41200	130	530
		Male	40400	137	482
15	105	Female	252000	752	442
		Male	354000	1170	386

abnormal clinical observations, macroscopic or microscopic findings related to the administration of MORAb-009 and the data indicated that the no observed adverse effect level (NOAEL) of MORAb-009 was greater than the seven doses of 15 mg/kg administered over 23 days.

Toxicokinetic analyses were performed and the results are summarized in Table 1. Systemic exposure of female and male monkeys to MORAb-009 increased proportionately with increasing dose. There were no gender differences at 2 mg/kg/h/dose (total dose = 14 mg/kg); however, exposures in males were greater than females at 15 mg/kg/h/dose (total dose = 105 mg/kg). Over a 7.5-fold dose range, AUC_{0-529.5h} values for females and males increased 6.12 and 8.76-fold, respectively, and C_{max} values for females and males increased 5.78 and 8.54-fold, respectively. The serum concentration levels of MORAb-009 at 30 minutes prior to and 30 minutes after the last infusion were greater than those after the second infusion, indicating a possible accumulation after repeated intravenous infusions. MORAb-009 was measurable in serum up to 1224 hours after starting the first infusion in the designated monkeys for the recovery period. MORAb-009 half-life was relatively long in designated female and male monkeys for the recovery period (341 and 285 hours, respectively). Overall, the pharmacokinetics of MORAb-009 followed the typical pattern of large biomolecules administered intravenously. The T_{max} occurred at the first time point measured after the intravenous infusion was complete (30 minutes post-infusion in this study). There was drug accumulation in the plasma following multiple infusions. The volume of distribution was initially equal to the plasma volume.

Discussion

To advance new therapeutic candidates for solid tumors expressing mesothelin, we conducted the preclinical evaluation of MORAb-009, a high affinity chimeric anti-mesothelin monoclonal antibody, and determined its suitability as an anti-tumor agent. Our results show that MORAb-009 is able to mediate biological responses *in vitro*, including inhibition of mesothelin-dependent cell adhesion and ADCC activity. In addition, MORAb-009 reduced tumor growth and enhanced the effect of chemotherapy in mice implanted with human tumor cells while exhibiting no adverse effects in non-human primates.

In order to detect possible cross-reactivity, MORAb-009 was applied to a human tissue panel that included all tissues recommended by the US Food and Drug Administration. The binding profile was essentially similar to that reported for other mesothelin-specific monoclonal antibodies, whereby reactivity to normal tissues was limited to mesothelium (data not shown). As part of a safety study, we attempted to determine the dose level at which potential toxicity may occur due to binding of MORAb-009. Since MORAb-009 does not bind to rodent tissues and its staining pattern in human and cynomolgus monkey

tissues was similar, only non-human primates were used to determine potential toxicity. The results of these studies indicated that the estimated no observed adverse effect level for MORAb-009 in cynomolgus monkeys was greater than the seven doses of 15 mg/kg administered over 23 days. The lack of toxicity observed could be explained by the limited mesothelin expression in normal tissues as evidenced by the IHC studies. Furthermore, although MORAb-009 was derived from a mouse antibody via chimerization with a human IgG1 backbone, it shares 82.6% amino acid sequence identity to a human IgG1κ. This may explain the lack of monkey anti-chimeric antibody responses observed.

Although MORAb-009 as a single agent had moderate anti-tumor activity against mesothelin-expressing tumor xenografts *in vivo*, this effect was markedly increased in combination with either gemcitabine or Taxol®. Presently, the mechanism(s) by which MORAb-009 enhances the anti-tumor effects of chemotherapeutic agents such as gemcitabine and Taxol® is unknown. One hypothesis is that binding of MORAb-009 leads to opsonization of tumor cells and their subsequent killing by cytotoxic immune cells, whereby this effect is additive or synergistic to that of chemotherapy. Mesothelin has been suggested to play a role in cell adhesion and we have shown that MORAb-009 can block mesothelin binding to CA125/MUC16 (Figure 5). Although we have no evidence that in our animal models tumor growth depends on cell adhesion, specifically on CA125/MUC16-mesothelin interaction, it is still possible that binding of MORAb-009 to mesothelin interferes with other tumor-host interactions, creating a condition whereby tumor cells are more susceptible to chemotherapy. Alternatively, gemcitabine and Taxol® can enhance MORAb-009 activity by improving tumor blood flow (20), facilitating the penetration and diffusion of MORAb-009 into the tumor. Two studies, one in mice and the other in humans, showed that Taxol® increased tumor uptake of radiolabeled antibody-interleukin 2 immunocytokine (21, 22). Regardless of the exact mechanism of the synergy between MORAb-009 and chemotherapy, this interaction could be useful in the clinic for the treatment of mesothelin-expressing cancers with MORAb-009 in combination with gemcitabine or Taxol®, two commonly used drugs for the treatment of pancreatic, ovarian, lung adenocarcinomas, and mesotheliomas.

In view of the encouraging preclinical data obtained from our studies, and to evaluate the safety profile of MORAb-009 in humans, we have initiated a Phase I clinical study enrolling patients with mesothelin-positive pancreatic, mesothelioma, non-small cell lung and ovarian cancers. Results obtained from this study will help guide the development of MORAb-009 alone or in combination with chemotherapy for the treatment of mesothelin-expressing cancers.

Abbreviations

FRα, folate receptor α

References

1. Chang K, Pastan I. Molecular cloning of mesothelin, a differentiation antigen present on mesothelium, mesotheliomas, and ovarian cancers. *Proc Natl Acad Sci U S A* 1996; **93**: 136-140. (PMID: 8552591)
2. Hassan R, Bera T, Pastan I. Mesothelin: a new target for immunotherapy. *Clin Cancer Res* 2004; **10**: 3937-3942. (PMID: 15217923)

3. Argani P, Iacobuzio-Donahue C, Ryu B, Rosty C, Goggins M, Wilentz RE, Murugesan SR, Leach SD, Jaffee E, Yeo CJ, Cameron JL, Kern SE, Hruban RH. Mesothelin is overexpressed in the vast majority of ductal adenocarcinomas of the pancreas: identification of a new pancreatic cancer marker by serial analysis of gene expression (SAGE). *Clin Cancer Res* 2001; **7**: 3862-3868. (PMID: 11751476)
4. Hassan R, Laszik ZG, Lerner M, Raffeld M, Postier R, Brackett D. Mesothelin is overexpressed in pancreaticobiliary adenocarcinomas but not in normal pancreas and chronic pancreatitis. *Am J Clin Pathol* 2005; **124**: 838-845. (PMID: 16416732)
5. Ordonez NG. Value of mesothelin immunostaining in the diagnosis of mesothelioma. *Mod Pathol* 2003; **16**: 192-197. (PMID: 12640097)
6. Chang K, Pastan I, Willingham MC. Isolation and characterization of a monoclonal antibody, K1, reactive with ovarian cancers and normal mesothelium. *Int J Cancer* 1992; **50**: 373-381. (PMID: 1735605)
7. Ordonez NG. Application of mesothelin immunostaining in tumor diagnosis. *Am J Surg Pathol* 2003; **27**: 1418-1428. (PMID: 14576474)
8. Hassan R, Kreitman RJ, Pastan I, Willingham MC. Localization of mesothelin in epithelial ovarian cancer. *Appl Immunohistochem Mol Morphol* 2005; **13**: 243-247. (PMID: 16082249)
9. Ho M, Bera TK, Willingham MC, Onda M, Hassan R, Fitzgerald D, Pastan I. Mesothelin expression in human lung cancer. *Clin Cancer Res* 2007; **13**: 1571-1575. (PMID: 17332303)
10. Miettinen M, Sarlomo-Rikala M. Expression of calretinin, thrombomodulin, keratin 5, and mesothelin in lung carcinomas of different types: an immunohistochemical analysis of 596 tumors in comparison with epithelioid mesotheliomas of the pleura. *Am J Surg Pathol* 2003; **27**: 150-158. (PMID: 12548160)
11. Rump A, Morikawa Y, Tanaka M, Minami S, Umesaki N, Takeuchi M, Miyajima A. Binding of ovarian cancer antigen CA125/MUC16 to mesothelin mediates cell adhesion. *J Biol Chem* 2004; **279**: 9190-9198. (PMID: 14676194)
12. Bera TK, Pastan I. Mesothelin is not required for normal mouse development or reproduction. *Mol Cell Biol* 2000; **20**: 2902-2906. (PMID: 10733593)
13. Hassan R, Bullock S, Premkumar A, Kreitman RJ, Kindler H, Willingham M, Pastan I. Phase I study of SS1P, a recombinant anti-mesothelin immunotoxin for targeted therapy of mesothelin expressing mesotheliomas, ovarian and pancreatic cancer [meeting abstract]. *J Clin Oncol* 2007; **25**: 3553.
14. Thomas AM, Santarsiero LM, Lutz ER, Armstrong TD, Chen YC, Huang LQ, Laheru DA, Goggins M, Hruban RH, Jaffee EM. Mesothelin-specific CD8(+) T cell responses provide evidence of in vivo cross-priming by antigen-presenting cells in vaccinated pancreatic cancer patients. *J Exp Med* 2004; **200**: 297-306. (PMID: 15289501)
15. Nicolaidis NC, Sass PM, Grasso L. Monoclonal antibodies: a morphing landscape for therapeutics. *Drug Dev Res* 2006; **67**: 781-789.
16. Chowdhury PS, Viner JL, Beers R, Pastan I. Isolation of a high-affinity stable single-chain Fv specific for mesothelin from DNA-immunized mice by phage display and construction of a recombinant immunotoxin with anti-tumor activity. *Proc Natl Acad Sci U S A* 1998; **95**: 669-674. (PMID: 9435250)
17. Chowdhury PS, Pastan I. Analysis of cloned Fvs from a phage display library indicates that DNA immunization can mimic antibody response generated by cell immunizations. *J Immunol Methods* 1999; **231**: 83-91. (PMID: 10648929)
18. Fransson J, Borrebaeck CA. The nuclear DNA repair protein Ku70/80 is a tumor-associated antigen displaying rapid receptor mediated endocytosis. *Int J Cancer* 2006; **119**: 2492-2496. (PMID: 16929494)
19. Gubbels JA, Belisle J, Onda M, Rancourt C, Migneault M, Ho M, Bera TK, Connor J, Sathyanarayana BK, Lee B, Pastan I, Patankar MS. Mesothelin-MUC16 binding is a high affinity, N-glycan dependent interaction that facilitates peritoneal metastasis of ovarian tumors. *Mol Cancer* 2006; **5**: 50. (PMID: 17067392)
20. Jain RK. Normalizing tumor vasculature with anti-angiogenic therapy: a new paradigm for combination therapy. *Nat Med* 2001; **7**: 987-989. (PMID: 11533692)
21. Holden SA, Lan Y, Pardo AM, Wesolowski JS, Gillies SD. Augmentation of antitumor activity of an antibody-interleukin 2 immunocytokine with chemotherapeutic agents. *Clin Cancer Res* 2001; **7**: 2862-2869. (PMID: 11555604)
22. Miers L, Lamborn K, Yuan A, Richman C, Natarajan A, DeNardo S, DeNardo G. Does paclitaxel (Taxol) given after (111)In-labeled monoclonal antibodies increase tumor-cumulated activity in epithelial cancers? *Clin Cancer Res* 2005; **11**: 7158s-7163s. (PMID: 16203816)
23. Jaffee EM, Schutte M, Gossett J, Morsberger LA, Adler AJ, Thomas M, Greten TF, Hruban RH, Yeo CJ, Griffin CA. Development and characterization of a cytokine-secreting pancreatic adenocarcinoma vaccine from primary tumors for use in clinical trials. *Cancer J Sci Am* 1998; **4**: 194-203. (PMID: 9612602)
24. Embuscado EE, Laheru D, Ricci F, Yun KJ, de Boom Witzel S, Seigel A, Flickinger K, Hidalgo M, Bova GS, Iacobuzio-Donahue CA. Immortalizing the complexity of cancer metastasis: genetic features of lethal metastatic pancreatic cancer obtained from rapid autopsy. *Cancer Biol Ther* 2005; **4**: 548-554. (PMID: 15846069)

Materials and methods

Cells

NCI-H226 and OVCAR-3 cells were purchased from the American Type Culture Collection (ATCC). IGROV-1 cells were obtained from the NCI cell collection. Panc 10.05 cells were previously described (23). Panc 3.27 and Panc 3.014 cells were generated in Dr. Elizabeth Jaffee's laboratory. A431-K5 cells were derived from A431, an epidermoid carcinoma cell line that has been stably transfected with a plasmid encoding mesothelin (6). A431-K5-TEM cells were derived in Dr. Grasso's laboratory from A431-K5 cells by transfection with an expression vector containing the full-length endosialin/TEM1 cDNA.

FACS analyses

Cells were harvested in cell dissociation solution (Invitrogen), washed, and resuspended in ice-cold PBS containing 1% FBS. Primary antibodies were added at 10 µg/mL and incubated for 1 hour on ice. Next, the cells were washed with PBS, incubated with FITC-conjugated goat-anti-human secondary antibody (Southern Biotech, Birmingham, AL) diluted to 1 µg/mL, washed as above and analyzed on an EasyCyte Flow Cytometer (Guava Technologies, Hayward, CA).

Immunohistochemistry

Samples of snap frozen pancreatic cancer were obtained from pancreaticoduodenectomy specimens or from tissues harvested in accordance with the Gastrointestinal Cancer Rapid Medical Donation Program at The Johns Hopkins Hospital (24). Five micron sections were cut from each tissue onto sterile glass slides and fixed in 100% ethanol for 1 minute, followed by rinsing in 1x TBS with Tween (TBST) for 5 minutes. Next, endogenous peroxidases were blocked by incubation with 3% hydrogen peroxide for 10 minutes followed by a second rinse in 1x TBST. Next, slides were incubated with a 0.5 µg/mL concentration of MORAb-009 antibody (Morphotek Inc., Exton, PA) diluted in 0.2 M Tris-HCL pH 7.5 for 1 hour at room temperature and again rinsed in 1x TBST for 5 minutes. Immunolabeling was detected using the LSAB+ Kit (DAKO) according to the manufacturer's instructions. Slides were counterstained in hematoxylin for 30 seconds and coverslipped. Staining patterns were evaluated by two of the authors (S.M. and C.I.D.) as negative (0), weakly positive (1+), positive (2+) and strongly positive (3+), and the percent labeling was evaluated on a 10-tiered scale ranging from 10% to 100%.

Internalization of MORAb-009

The human cell lines NCI-H226 and IGROV-1 were cultured in complete RPMI 1640 medium (1 mM sodium pyruvate, 5 U/mL penicillin, 5 µg/mL streptomycin, 2 mM L-glutamine) containing 10% FBS (Invitrogen) at 37°C, in a humidified atmosphere containing 5% CO₂. One day prior to addition of antibodies, cells were collected by trypsinization and plated at a density of 500 cells/well in a 96-well tissue culture plate (Nalgene Nunc) in complete RPMI 1640 medium. MORAb-009, polyclonal, saporin-conjugated goat anti-human antibodies (Hum-ZAP, Advanced Targeting Systems) or control antibodies were mixed in various ratios and incubated for 30 minutes at room temperature, prior to addition to cultured cells. Cells were incubated at 37°C, in a humidified atmosphere containing 5% CO₂, for 5 days prior to harvesting. Relative changes in cell numbers were quantified by the sulforhodamine B method. Briefly, cells were killed and fixed for one hour on ice by addition of ice-cold 100% trichloroacetic acid (TCA) directly into the growth medium, to a final concentration of 10%. TCA was removed by washing fixed cells 5 times using distilled, deionized water, and allowed to dry in open atmosphere. 100 µL of a solution of 0.4% sulforhodamine B in distilled, deionized water were added to each well, and incubated for 10 minutes. The wells were washed 4 times using 1% acetic acid and allowed to dry in open atmosphere. The resulting precipitate was dissolved in 100 µL of 10 mM Tris-Cl pH 8.0, and the absorbance at 540 nm was measured with a microplate reader. Relative cell numbers were determined by interpolation from a standard curve constructed by measuring absorbance of known cell numbers from each cell line, deposited in the bottom of a

96-well tissue culture plate. Results were expressed as % growth, by comparison to untreated cells.

ADCC assay

Donor peripheral blood mononuclear cells (PBMCs) used as effector cells were thawed and kept overnight in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal calf serum. The cells were resuspended in medium at a concentration of 10⁷ cells/mL. The tumor cells used as target cells were detached from the culture flask and 10⁶ cells in 100 µL fetal calf serum were labeled with 100 µCi (3.7 MBq) ⁵¹Cr for 2 hours at 37°C. Alternatively, lysing of cells was monitored by a standard lactate dehydrogenase (LDH) release assay following the manufacturer's instructions (Roche Applied Science, Indianapolis, IN). Cells were washed three times with 5 mL of medium and resuspended in medium at a concentration of 10⁵ cells/mL. Fifty microliters of the tumor cells were seeded in V-bottom 96-well culture plates. Cells were then incubated with 50 µL medium containing MORAb-009 or control antibody at concentrations indicated in the text. After 30 minutes of incubation at 37°C, 50 µL of the PBMCs were seeded in V-bottom 96-well culture plates at various target-effector cell ratios (1:0, 1:25, 1:50 and 1:100) and the plates were further incubated for 18 hours at 37°C. The release of ⁵¹Cr in the supernatant was determined in a gamma counter. Each measurement was carried out in triplicate.

CA125/MUC16-mesothelin interaction assay

OVCAR-3 cells (1 x 10⁵) were seeded in triplicate in microplates, incubated overnight at 37°C/5% CO₂ and allowed to form a monolayer. The following day, A431-K5-TEM cells (mesothelin+/TEM1+) were harvested and loaded with Calcein AM cell dye (Invitrogen Corp., Carlsbad, CA) as per the instruction manual. Labeled cells (2 x 10⁵) were pre-incubated for 1 hour at 4°C with MORAb-009 or control antibodies at a final concentration indicated in the figure legends. Both normal human polyclonal IgG (Jackson ImmunoResearch, WestGrove, PA) and anti-TEM1 antibody (Morphotek Inc., Exton, PA) were included to control for the specificity of the MORAb-009 inhibition of the mesothelin-MUC16 interaction. The OVCAR-3 monolayers were washed once with 200 µL 10% complete RPMI (cRPMI) and the cells/antibody mixtures added to triplicate wells for 45 minutes at 4°C. Wells were gently washed five times with 200 µL PBS by inverting the plate on paper towels. Cell adherence and its inhibition by MORAb-009 were quantitated using the MetaMorph Fluorescence Imaging System (ver.6, Molecular Dynamics, Downingtown, PA).

In vivo efficacy studies

Gemcitabine and Taxol® were provided by the Division of Veterinary Resources (NIH). A431-K5 cells were grown in DMEM with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/mL penicillin G, 100 µg/mL streptomycin and 750 µg/mL G418. Tumor experiments evaluating MORAb-009 in combination with gemcitabine were conducted using A431-K5 xenografts in nude mice. Four to six week old female athymic nude mice (National Cancer Institute-Frederick Animal Production Area, Frederick, MD) were housed in microisolator cages for the course of the experiment. The research protocol was approved and the mice were maintained as per institutional guidelines of the NIH. Two million A431-K5 cells were inoculated subcutaneously into the right flank of the mice. Tumor dimensions were determined using calipers and the tumor volume (mm³) was calculated by the formula: length

$\times (\text{width})^2 \times 0.4$. Treatment was initiated when tumors reached approximately 50 mm³ in size. The different treatment regimens included: gemcitabine (80 mg/kg) alone via i.p. injection on days 7, 9 and 11 after tumor inoculation; MORAb-009 or Rituximab (50 mg/kg) alone given i.v. on days 7, 9 and 11 after tumor inoculation; gemcitabine (80 mg/kg) i.p. on days 7, 9 and 11 in combination with either MORAb-009 or Rituximab (50 mg/kg) i.v. on days 7, 9, 11, 13, 15 and 17 after tumor inoculation. Mice were sacrificed when the tumors reached approximately 500 mm³. To test the *in vivo* activity of MORAb-009 in combination with Taxol®, the same A431-K5 tumor xenograft model was used. On day 4 after tumor cell inoculation when the tumor size was approximately 60 mm³, mice ($n = 7$ per treatment group) were treated with either Taxol® 50 mg/kg i.p. on day 4, MORAb-009 50 mg/kg i.v. on days 4, 6 and 8 alone or the combination of Taxol® 50 mg/kg on day 4 and MORAb-009 50 mg/kg i.v. on days 4, 6, 8, 10, 12 and 14. Mice were sacrificed when the tumors reached approximately 500 mm³.

Non-human primate toxicity study

After preliminary dose-ranging studies, a 23-day intravenous infusion toxicity study in cynomolgus monkeys with a 28-day recovery period was conducted under Good Laboratory Practice (GLP). At the saline control and 15 mg/kg dose levels, three monkeys per sex were dosed with MORAb-009 twice per week over 23 days (seven total doses). At the 2 mg/kg dose level, two monkeys per sex were dosed at the same frequency. Two days after the last dose, two monkeys per sex were euthanized and their necropsy carried out. The remaining control and high dose monkeys in the respective recovery groups were observed for 28 additional days before euthanasia and necropsy. Clinical evaluations of the monkeys included the following: physical examinations pre- and post-dosing; daily clinical observations, including mortality checks; body weight measurements prior to study start, and twice weekly throughout the study; daily food consumption estimates beginning one week prior to treatment throughout the study; and hematology, coagulation, clinical chemistry and urinalysis measurements prior to study start and at study termination. Postmortem evaluations included the following: complete macroscopic examinations on all monkeys; selected organ weights; and complete histopathology examination. Blood was drawn before and after each dose, as well as during the recovery period for the determination of the toxicokinetics of MORAb-009 and for the detection of the possible generation of monkey anti-chimeric antibodies, which were determined by using ELISA.

Contact

Address correspondence to:

Luigi Grasso
210 Welsh Pool Road
Exton, PA 19341
USA
E-mail: grasso@morphotek.com

REVIEWS

Antibody–Drug Conjugates: Linking Cytotoxic Payloads to Monoclonal Antibodies

Laurent Ducry* and Bernhard Stump

Lonza Ltd, CH-3930 Visp, Switzerland. Received May 4, 2009; Revised Manuscript Received July 7, 2009

Antibody–drug conjugates (ADCs) combine the specificity of monoclonal antibodies (mAbs) with the potency of cytotoxic molecules, thereby taking advantage of the best characteristics of both components. Along with the development of the mAbs and cytotoxins, the design of chemical linkers to covalently bind these building blocks is making rapid progress but remains challenging. Recent advances have resulted in linkers having increased stability in the bloodstream while allowing efficient payload release within the tumor cell.

INTRODUCTION

After cardiovascular diseases, cancer represents the second most common cause of death in the Western world and is a major area of focus for the pharmaceutical industry. Surgery and radiotherapy are generally used when a tumor is localized to a certain tissue, but chemotherapy is needed when metastasis has occurred. Despite extensive research, most anticancer drugs have nonspecific toxicity. By targeting the cell cycle and thereby killing rapidly proliferating cells, they do not explicitly discriminate between healthy and tumor tissues and only gain a limited selectivity for malignant cells. Such cytotoxic drugs have a narrow therapeutic window, which limits their efficacy and results in severe side effects. Due to a lack of selectivity, drug concentrations that would eradicate the tumor can often not be used. In addition, tumors can develop resistance against anticancer drugs after prolonged treatment. Therefore, achieving improved tumor selectivity through targeting of cytotoxic drugs to the cancer cells is needed.

A promising approach to achieving a more selective treatment is targeted prodrug therapy (1). Antibody–drug conjugates (ADCs) are ideal candidates for such prodrugs. ADCs are monoclonal antibodies (mAbs) linked to cell-killing drugs. Thanks to their high binding specificity for tumor-specific antigens, mAbs can be used as vehicles to target cell-killing payloads to tumor cells. Unique or overexpressed, tumor-specific antigens can be found in a wide range of human tumor cells (2). Some mAbs have the ability to recognize and specifically bind to these tumor-associated antigens. They can be used as single agents for the treatment of cancer through binding to cancer-cell-specific antigens and induction of an immunological response against the target cancer cell (3). However, therapeutic efficacy is often limited by the extent to which the antibody leads to cell death. Monoclonal antibodies are extremely discriminating for their targets but sometimes therapeutically ineffective on their own. The insufficient efficiency of most naked mAbs in cancer therapy has been circumvented by arming the immunoglobulin with radioactive isotopes (4) or cytotoxic drugs (5–7) (Figure 1), yielding highly specific ADCs.

ADCs can be viewed as sophisticated delivery systems for antitumor cytotoxic drugs. The mAb guides the toxin precursor

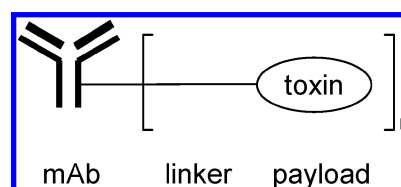


Figure 1. Schematic representation of an antibody–drug conjugate.

to the target cancer cell, where the prodrug can be converted chemically and/or enzymatically to the parent drug and unfold its cytotoxic activity. The ADC is exposed to different conditions on its journey from the blood vessels to the molecular target in the tumor tissue. The mode of action at cellular or molecular level is complex, with each step bringing its own challenges:

a. Circulation. The ADC must behave like a naked antibody when circulating in the plasma. In particular, the linker must be stable in the bloodstream to limit the damage to healthy tissue. Decomposition or decay would release the cytotoxin before being delivered to the target site.

b. Antigen Binding. It is necessary that the conjugated mAb retains high immunoaffinity. Attaching the cytotoxic compound to the mAb must thus not disturb its binding specificity.

c. Internalization. A sufficient intracellular concentration of the drug must be achieved. This is challenging because antigen targets on cell surfaces are often present in limited numbers and the internalization process for antigen–antibody complexes is frequently inefficient.

d. Drug Release. Once internalized, the ADC has to efficiently release the original cytotoxic drug in its active form inside the tumor cell.

e. Drug Action. The inherent potency of the released drug must be sufficient to kill the tumor cell, even at low concentration. To achieve significant cytotoxicity, use of very potent drugs with subnanomolar IC_{50} (as free drug) becomes necessary. It has been shown that compounds found to be too toxic when tested as a stand-alone chemotherapy agent are suitable candidates for ADC payloads. These toxins can be 100 to 1000 times more cytotoxic than traditional anticancer agents.

The molar loading of the drug on the mAb can be tuned to achieve the desired potency. Because of points (c) and (d), linkage of several drugs is generally necessary to achieve significant cytotoxicity. However, if too many cytotoxic molecules are attached

* laurent.ducry@lonza.com.

Chart 1

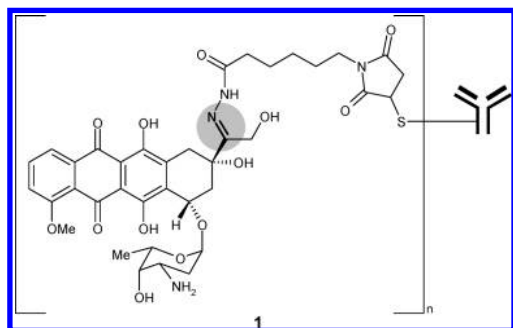
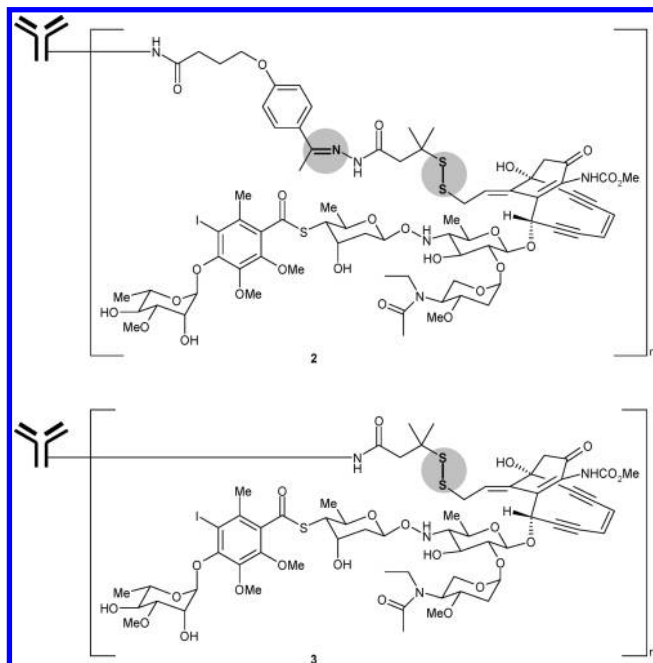


Chart 2



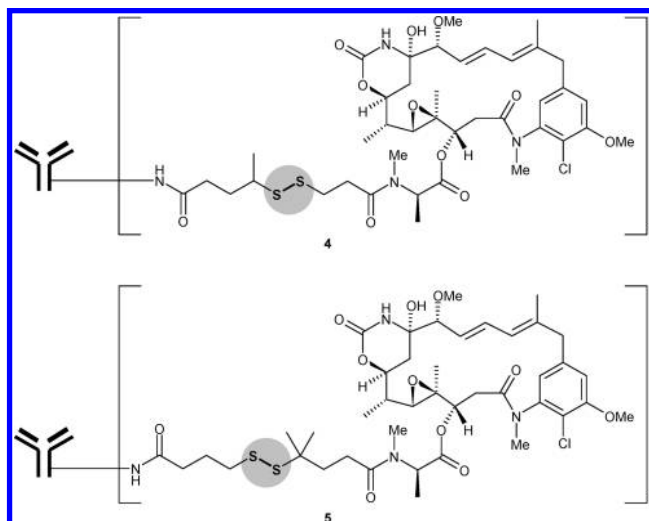
to the antibody, the body may recognize the conjugate as a damaged form of the protein and quickly clears it from the system, proving points (a) and/or (b) to be problematic. The extent of drug substitution may also affect pharmacokinetics, so that most of the time 2–4 drugs per antibody give the best therapeutic window (8, 9). To avoid the interference with the antigen recognition, the drugs are ideally linked at the Fc or constant region of the mAb that does not participate in binding to the antigen (point (b)). To regain the full cytotoxic potential of the drugs, cleavable linkers releasing the drug molecule at the target site in unmodified form were developed to address point (d), although these also affect the stability of the construct. Therefore, the right balance between plasma stability and efficient drug release at the target cell has to be found.

The insights gained during ADC development within the past decade have resulted in a number of new strategies and drugs (4–7). This review focuses on linker technologies used to attach low molecular weight cytotoxic drugs to mAbs. The linker is a small but central part of ADCs and as such accounts for stability in circulation, favorable pharmacokinetic properties, and efficient release of toxic agent at the tumor site.

CHEMICALLY LABILE LINKERS

Selective linker cleavage and payload release is based upon the differential properties between the plasma and some cytoplasmic compartments. Linkers were chosen that were stable in the blood's neutral pH environment but were getting cleaved

Chart 3



once the ADC entered the lower pH environment found inside a cell. The focus in the early days of ADC development was on acid-cleavable hydrazone linkers that were relatively stable at neutral pH (bloodstream pH 7.3–7.5), but were undergoing hydrolysis once internalized into the mildly acidic endosomes (pH 5.0–6.5) and lysosomes (pH 4.5–5.0).

In the 1990s, Bristol-Myers Squibb scientists covalently linked doxorubicin, an intercalating agent that blocks DNA replication and is used as chemotherapeutic agent, to the humanized mAb BR96 (10). Doxorubicin was connected over a (6-maleimidocaproyl)hydrazone linker to cysteine residues of the Lewis-Y specific mAb. The preclinical activities of BR96-doxorubicin (**1**, Chart 1) were remarkable, but despite the use of eight drugs per mAb molecule, the amount of conjugate needed to achieve these effects *in vivo* were high (>100 mg/kg). This was presumably due to the relatively low potency of doxorubicin (IC_{50} of 0.1–0.2 μ M for human carcinoma lines (11), whereas subnanomolar activities are now typically seen for ADC payloads). Moreover, the half-life of the drug in the blood was approximately 43 h, which is short compared to the half-lives of several days to weeks of the naked BR96 mAb in humans. The compound later failed to show sufficient clinical efficacy.

The first and so far sole mAb linked to a cytotoxic payload that has been given regulatory approval is Wyeth's gemtuzumab ozogamicin (**2**, Mylotarg; Chart 2) (12–14). The drug was approved by the US Food and Drug Administration (FDA) in 2000 for use in patients over 60 suffering from relapsed acute myelocytic leukemia (AML), the most common form of leukemia in adults. Gemtuzumab ozogamicin (**2**) consists of *N*-acetyl- γ -calicheamicin (an enediyne antibiotic that binds to the minor groove of DNA and causes double-strand breaks leading to apoptosis) covalently attached to humanized anti-CD33 IgG4 κ antibody (hP67.6) via a bifunctional linker. The 4-(4-acetylphenoxy)butanoic acid moiety allows for attachment to surface-exposed lysines of the antibody over an amide bond and forms an acyl hydrazone linkage with *N*-acetyl- γ -calicheamicin dimethyl hydrazide. Upon internalization of the ADC, the calicheamicin prodrug is released by hydrolysis of the hydrazone in lysosomes of the CD33-positive target cells. The hydrolyzability of an unconjugated intermediate at 37 °C over 24 h increased from 6% at pH 7.4 to 97% at pH 4.5 (15). The enediyne drug then gets activated by reductive cleavage of the disulfide bond. This disulfide linkage has been stabilized by two methyl groups to prevent premature release of calicheamicin by circulating reduced thiols, such as glutathione. In most preclinical models, the hydrazone linkage produced ADCs with higher potency than the corresponding amide-bearing conjugate **3**, providing evidence that with this mAb the disulfide alone is insufficient for

Scheme 1. Presumed Release Mechanism of MMAE (7) from ADC 6 Incorporating a Valine-Citrulline Peptidic Linker

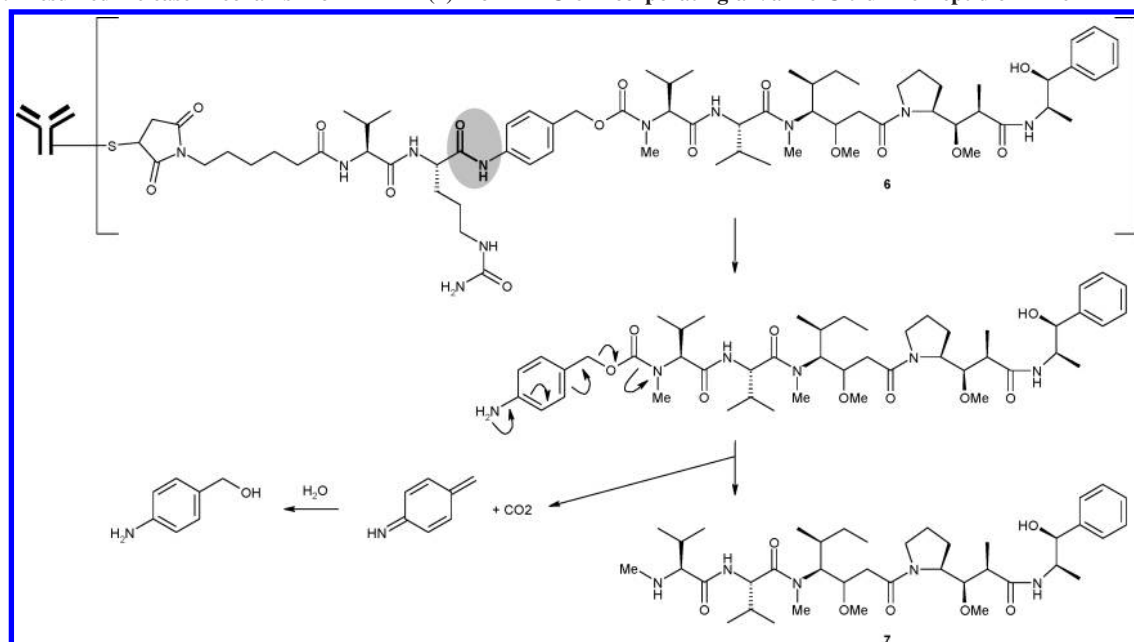


Table 1. Compared Stabilities of Enzyme-Labile and Chemically Labile Linkers in ADCs with MMAE Conjugated to cBR96 mAb

ADC	Linker	Half-live mouse plasma	Half-live human plasma	Maximum tolerated dose
	Valine-citrulline	30 days*	230 days*	30 mg/kg
	Phenylalanine-lysine	12.5 days*	80 days*	n/a
	Hydrazone	2.1 days	2.6 days	15 mg/kg
* projected half-life				

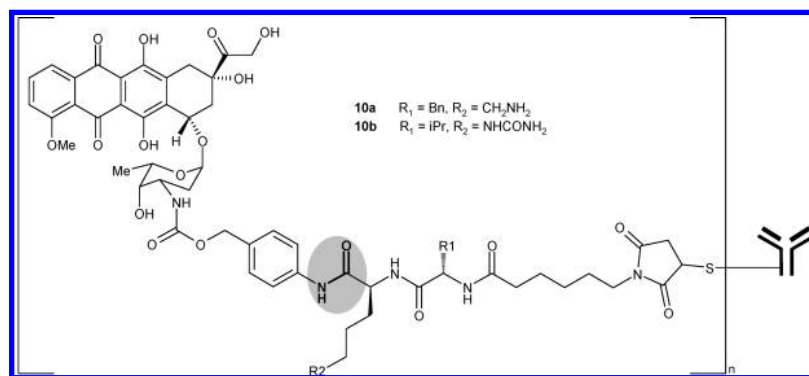
efficient release of the drug in the target cell. Interestingly, with the murine mAb CTM01, ADC **3** showed activities equal to or even greater than that of the corresponding hydrazone conjugate **2** in several *in vitro* and *in vivo* tumor models, revealing that this conclusion is not general (16). In clinical trials, however, compound **3** had only limited evidence of activity.

Mylotarg has met limited successes due to a narrow therapeutic window. The linker technology based on pH-dependent release mechanism is possibly not sufficiently stable and too much of the drug is being released in the bloodstream, as pharmacokinetic data have shown that the mean half-life of Mylotarg is 72 h (15). Nonetheless, the development of CMC-544 (inotuzumab ozogamicin), which is a humanized anti-CD22 mAb identically attached to *N*-acetyl- γ -calicheamicin dimethyl

hydrazide via the acid-labile 4-(4'-acetylphenoxy)butanoic acid linker is ongoing at Wyeth. Although this ADC is closely related to Mylotarg, good stability was shown in both human plasma and serum (rate of hydrolysis of 1.5–2%/day over 4 days) (17, 18).

Disulfide bonds can be an alternative to acid-labile hydrazone linkers. In this case, the release is attributed to the high intracellular concentration of glutathione. This thiol-containing tripeptide is present in micromolar concentrations in the blood, whereas its concentration in the cytoplasm is in the millimolar range (up to 1000-fold higher). This is especially true for tumor cells, where irregular blood flow leads to hypoxic state (decreased oxygen level), resulting in enhanced activity of reductive enzymes and therefore even higher glutathione concentrations. In the absence of sulfhydryl groups, disulfide

Chart 4



bonds are thermodynamically stable and provide reasonably good stability in the bloodstream.

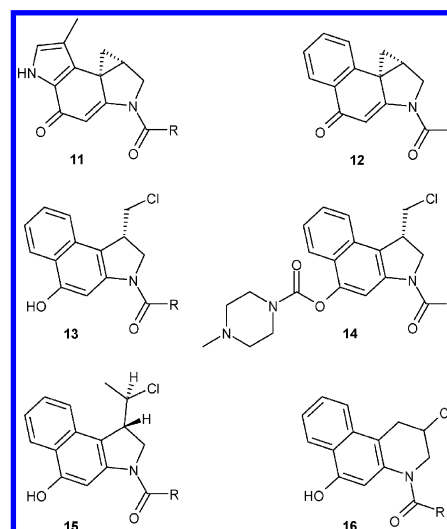
An example of an intracellularly cleavable disulfide-based linker was already discussed above (see “amide” conjugate **3**). Other examples are taxoid ADC payloads, conjugated via a disulfide-bearing 4-mercaptopentanoate linker to mAbs directed against the epidermal growth factor receptor (19). However, the most significant examples are ADCs where the immunoglobulin is connected over the same type of linker to ImmunoGen’s drugs DM1 or DM4 (20–22). These potent thiol-containing maytansine analogues are antimitotic agents that bind to tubulin and inhibit microtubule assembly. After internalization of the ADC via antigen-mediated endocytosis and delivery to lysosomes by vesicular trafficking, the mAb is believed to be degraded, affording lysine derivatives linked to the toxin (22). Further intracellular modifications like cleavage of the disulfide linker through a disulfide exchange, and finally thiol methylation presumably catalyzed by intracellular methyltransferases, generate potent derivatives of DM1 or DM4 (22). These methylated drugs are uncharged, enabling them to pass back out through cell membranes to broaden their attack; the drug not only kills from inside the cell, but also knocks out adjacent cells within the tumor that are not targeted by the antibody (23). The linker of compound **4** (Chart 3) nevertheless proved to be relatively unstable *in vivo* (half-life of 47 h in mice) (24). Replacement of DM1 by DM4 structurally leads to the introduction of two methyl substituents on the carbon atom geminal to the disulfide bond, thereby limiting the accessibility for reducing agents. Consistent with these considerations, ADC **5** exhibits an increased half-life of 102 h in mice.

ENZYME-LABILE LINKERS

Chemically labile linkers often suffer from limited plasma stability. An alternative approach to achieve better control of the drug release involves linking a drug to an antibody via a peptide linkage. The free drug can be specifically cleaved from the carrier by the action of lysosomal proteases (e.g., cathepsin or plasmin) present at elevated levels in certain tumor tissues (25). Peptidic bonds are expected to have good serum stability, as proteases are normally not active outside cells because of unfavorable pH conditions and inhibition by serum protease inhibitors.

Seattle Genetics is using a maleimido-containing dipeptide linker to conjugate cysteine residues of mAbs with monomethyl auristatin E (MMAE, **7**) and F (MMAF), two auristatin derivatives that bind to tubulin and inhibit microtubule assembly. A valine-citrulline linker was designed to provide high plasma stability (half-lives in mice and monkey of 6.0 and 9.6 days, respectively) as well as a cleavage site for proteases (26). Efficient hydrolysis of the dipeptide linker by cathepsin B has been established *in vitro*. After enzymatic

Chart 5



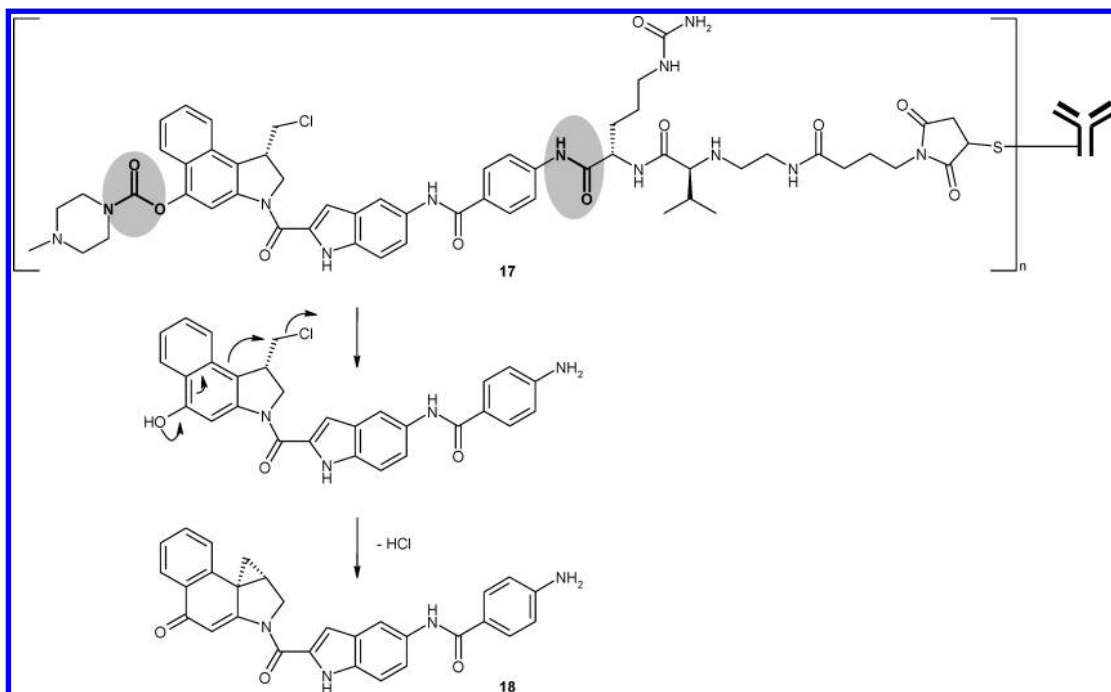
cleavage, 1,6-elimination of the strongly electron-donating 4-aminobenzyl group will occur, releasing MMAE in its active form (Scheme 1). This self-eliminating spacer is needed to spatially separate the drug from the site of enzymatic cleavage (27). Otherwise, the bulky payload has a negative influence on the kinetics of the peptide hydrolysis, as the access to the enzyme’s active site can be affected.

Comparison of plasma stabilities of ADCs bearing enzyme-labile valine-citrulline or phenylalanine-lysine linkers (**6** and **8**, respectively) with ADC **9** equipped with a chemically labile hydrazone linker connecting MMAE with the cBR96 mAb showed impressively the advantage of the peptide linkage (Table 1): in human plasma, the ADC **6**, featuring a peptidic valine-citrulline-derived linker, exhibits an almost 100-fold increased stability compared to its hydrazone relative **9** and the *in vitro* specificity was increased (26). The peptidic linker has a half-life of 6 days in mice compared to 2 days for the hydrazone linker (3-fold improvement), resulting in a lower *in vivo* toxicity (28). Several ADCs incorporating a peptidic linker are in clinical development, with the most advanced being cAC10-vcMMAE (SGN-35) for CD30-positive hematologic malignancies (26, 29, 30).

In addition to *N*-terminus linked MMAE and MMAF, attachment of dipeptide linkers at the *C*-terminus was studied as a way to vary the rate of drug release and thus ADC potency and tolerability (26, 31). For MMAF, it could be shown that linking the mAb over a dipeptide linker to the *C*-terminus of the drug can lead to further improvement of the therapeutic window.

The superiority of enzyme-labile linkers was also shown for doxorubicin derivatives conjugated to mAbs (11, 32). With this

Scheme 2. Presumed Release Mechanism of CBI-Containing Drug 18 from ADC 17



strategy, the immunological specificity of BR96 mAb–peptide–doxorubicin conjugates **10a** and **10b** (Chart 4) was significantly improved compared to that of the corresponding hydrazone-based conjugate **1** (11), demonstrating again the importance of the linker structure for the ADC properties.

Peptide-containing linkers have been successfully used with CC-1065-derived toxins. CC-1065, duocarmycins, and other cyclopropapyrrolind-4-one (CPI, **11**; Chart 5) derivatives are potent minor-groove binding DNA alkylating agents. According to Boger et al., cyclopropabenzindol-4-one analogues (CBI, **12**) are chemically more stable, biologically more potent, and synthetically more accessible than their parent compounds incorporating the natural CPI alkylation subunit (33). In analogy to a duocarmycin prodrug (34), the seco-CBI precursor **13** was developed. This compound, which was postulated to show improved pharmacokinetics, will provide the CBI analogue **12** via Winstein cyclization (35). A further improvement was achieved by protecting the phenolic hydroxyl group with an enzyme-labile carbamate, as in derivative **14**, decreasing the toxicity of the prodrug while increasing its water solubility by introduction of an additional basic nitrogen (36).

Using their UltiMAB technology, Medarex conjugated compound **14** to a mAb via a peptidic linker (37). Conceptually, this approach is a double prodrug strategy. It does not only improve the drug's therapeutic efficacy by conjugating it to a tumor-specific mAb, but additionally employs a prodrug of the cytotoxic agent that has reduced potency and thus minimizes side effects before being released intracellularly by carboxyesterases. The two activating steps needed to trigger the release of the active drug (Scheme 2) may lead to an enlarged therapeutic window.

Tietze et al. have reported that the CBI prodrugs **15** and **16** exhibit lower toxicities due to the reduced reactivity of the secondary chloride compared to the primary chloride present in **13** and **14** (39–41). Such derivatives with secondary chlorides (see, for example, ADC **19**; Chart 6) are used in Syntarga's SpaceLink conjugation technology (42). To our knowledge, no data are yet published on the linker stability.

To extend its ADC technology to drugs with a complementary mode of action, Seattle Genetics has developed

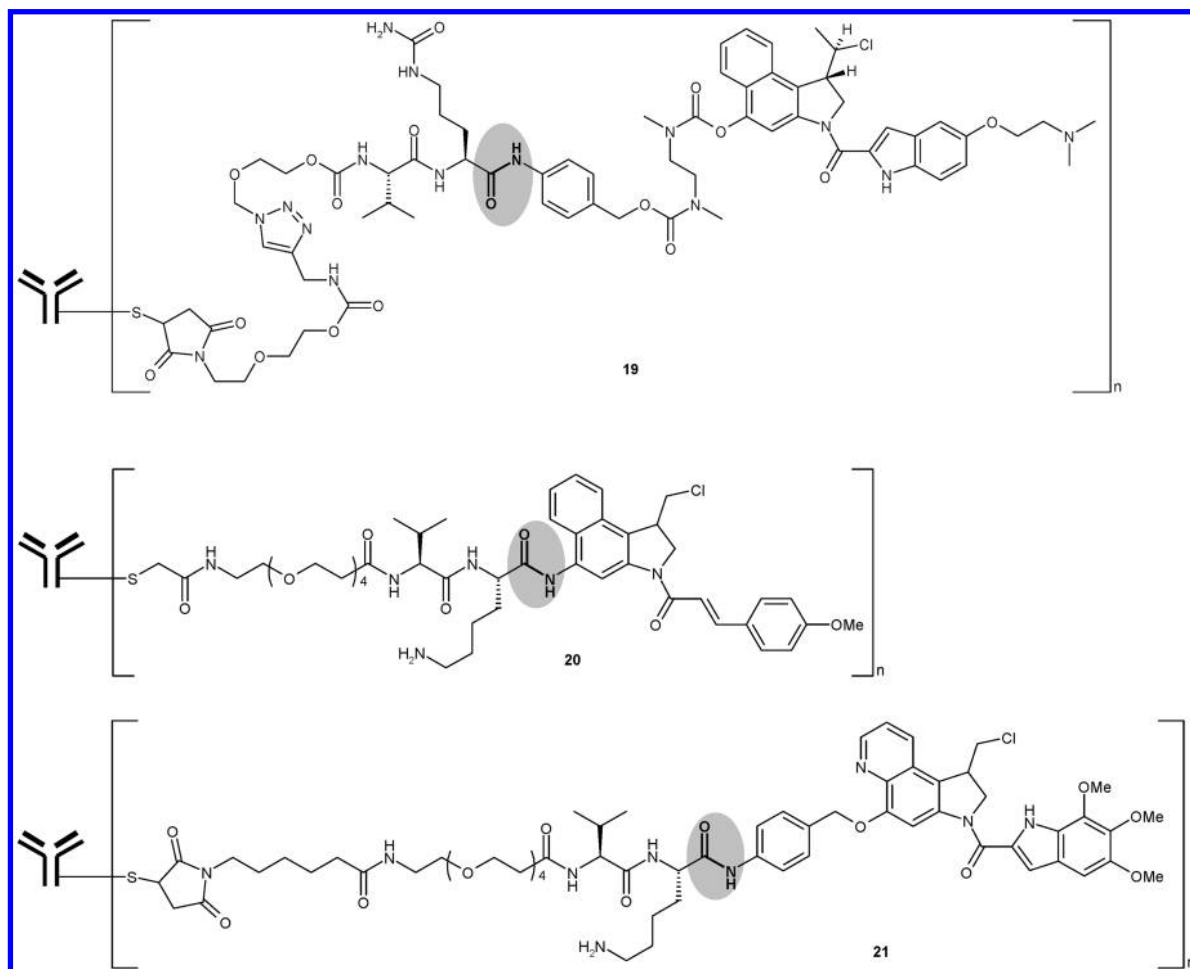
ADCs **20** and **21** (Chart 6) containing an amino-CBI and a hydroxy aza-CBI payload, respectively (43). Because of the hydrophobicity of this class of molecules, attention was focused on developing hydrophilic peptide-linker derivatives that prevented aggregation. This was achieved by use of a more hydrophilic valine-lysine sequence (compared to valine-citrulline as in ADC **6**) and incorporating a tetra(ethylene glycol) unit between the mAb and the peptide linker. The direct attachment of the linker to the amine of the CBI building block (as in **20**) or over a self-immolative *p*-aminobenzyl spacer to the corresponding hydroxyl group of the aza-CBI toxin (as in **21**) prohibits the spontaneous formation of the active toxin in the plasma. Only the enzymatic cleavage of the linker after internalization of the ADC into the cancer cell triggers the release of the prodrug that can now be transformed into the DNA-alkylating cyclopropyl derivative via a Winstein cyclization.

Another type of enzyme-labile linker was recently reported by Seattle Genetics (44). In this case, a carbohydrate moiety serves as the cleavage site. Its hydrophilic nature should limit aggregation. Interestingly, the β -glucuronide moiety in ADC **22** is not directly linking the ADC with the payload. Nevertheless, cleavage by β -glucuronidase triggers the 1,6-elimination of the spacer liberating the free drug **23** (Scheme 3). This carbohydrate motif was also used in ADCs bearing Camptothecin analogues (45).

NONCLEAVABLE LINKERS

ImmunoGen has reported the fortuitous discovery of a noncleavable thioether bond to link DM1 to mAbs (22, 46). Derivative **24** was actually prepared as a control experiment, but surprisingly, this noncleavable thioether linked ADC also proved very potent. The release mechanism is believed to occur via internalization of the ADC followed by degradation of the mAb component in the lysosome, resulting in the release of the maytansinoid drug **25** still attached via the linker to a lysine residue. This chemical modification of the drug did not diminish its cytotoxic potential. This form of the drug is, however, charged and presumably not able to diffuse into neighboring cells. Hence, it cannot kill adjacent tumor cells

Chart 6



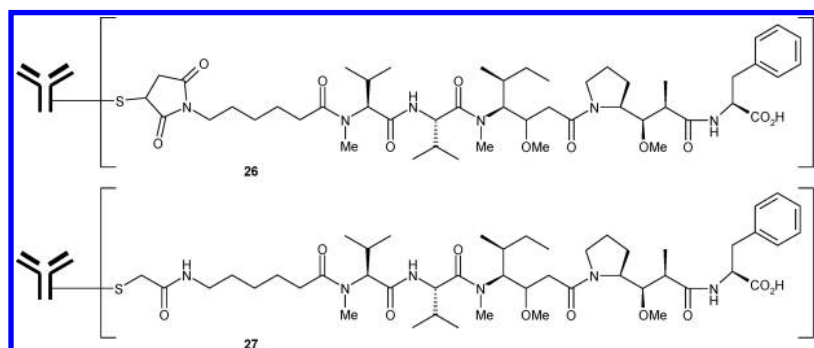
that do not express the target antigen (antigen-negative cells). Besides this by-stander effect (23), there is an additional reason why cleavable linkers may have a broader efficacy. Although internalization is often the common initial activation process for both cleavable and noncleavable linkers (22), ADCs with cleavable linkers may also be active even when they are poorly internalized (47). One possible explanation is that, when the linker is cleaved in extracellular space, the free drug subsequently permeates the cell to reach its target. This mechanism is not operating for ADCs incorporating noncleavable linkers, which must be internalized and degraded within the cell to become active. They are thus dependent on the biology of the target cell.

The *in vivo* stability of the thioether-linked ADC **24** proved, with a half-life of 134 h in mice, superior to that of compounds **4** and **5** (47 and 102 h, respectively). ADCs with

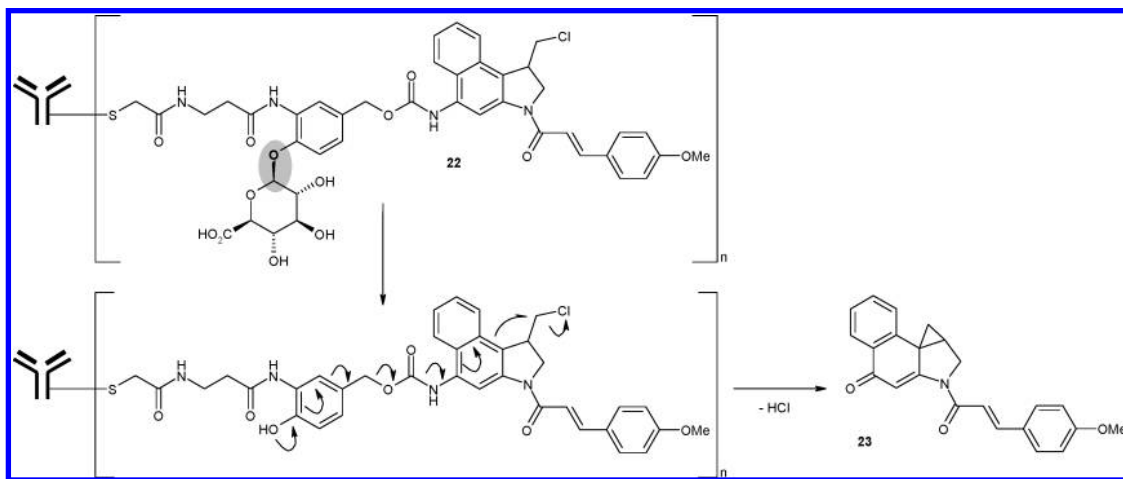
noncleavable linkers were found to be better tolerated (47), presumably resulting in an improved therapeutic index. The use of noncleavable linkers has thus become an important feature of ImmunoGen TAP (tumor-activated prodrug) conjugation technology. This is best exemplified by the promising clinical results obtained by Genentech's trastuzumab-DM1 (T-DM1) for HER2-positive metastatic breast cancer (48).

Seattle Genetics made the same finding with MMAF, namely, that the cleavable dipeptide linker (as in **6**) could be replaced by a noncleavable thioether bond (as in ADC **26**; Chart 7) while retaining the *in vivo* activity (49). Mass spectrometry showed that the released drug was a cysteine-adduct of MMAF, presumably resulting from mAb degradation within lysosomes as proposed for maytansinoid conjugate **24**. MMAE, although closely related structurally, was not

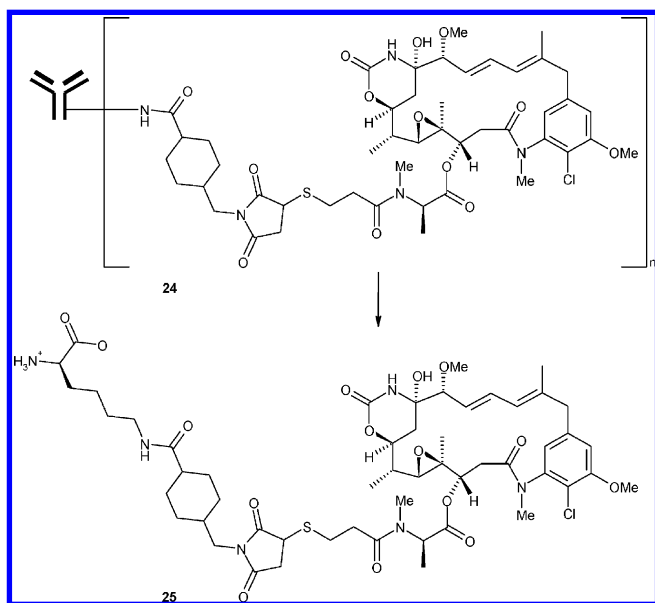
Chart 7



Scheme 3. Postulated Release Mechanism of CBI-Containing Drug 23 from ADC 22



Scheme 4. Release of Maytansine Drug-Derivative 25 through Lysosomal Degradation of ADC 24



active when attached in this manner. Only MMAF can sustain significant modification to the *N*-terminal position and still retain its potency. Recently, it was shown that a slow transfer of the drug from the ADC to albumin cysteines in the plasma occurs with alkyl-maleimide ADCs (50). To counteract this effect, the linker stability was enhanced by replacing the maleimide (as in ADC 26) with an acetamide functionality (as in ADC 27). This type of ADC led to no apparent degradation in the bloodstream for up to 14 days.

CONCLUSION

A promising approach to improve the potency of antibody-based cancer therapies is to conjugate the mAb with cytotoxic chemotherapeutic drugs. In the foreseeable future, ADCs will eventually fulfill the promise of specific delivery of cytotoxic drugs to tumor cells (targeted therapy), thus avoiding the dose-limiting toxicity of chemotherapy that occurs as a result of its effects on normal cells. The linker technology profoundly impacts ADC potency, specificity, and safety. Early ADC linkers were commonly derived from acid-labile hydrazones, which were designed to be cleaved inside target cancer cells, but inevitably underwent cleavage at nontarget sites. Newer linkers feature hindered disulfides and peptidic

moieties that have achieved greater *in vivo* stability. To spare nontarget tissues from chemotherapeutic damage, noncleavable linkers were recently developed. They are thus far the most stable linkers used in ADCs. This recent development may shift some of the future challenge in ADC development from the linker technology toward finding cytotoxic drugs that retain their potency when bearing a covalently attached linker. The improved linker stability and reduced toxicity, however, come together with greater target dependence than in the case of cleavable linkers. ADCs with noncleavable linkers must be internalized and degraded within the cell, whereas compounds with cleavable linkers may be active against targets that are poorly internalized through extracellular drug release and drug entry into tumor cells. Similarly, killing of bystander antigen-negative cells through targeting of antigen-positive cells (collateral toxicity) is presumably only possible with cleavable linkers. The Lonza ADC-team thus believes that no general linker design exists for all ADCs or cancer types. Despite significant progress, each mAb must be examined separately in order to optimize a specific delivery system.

LITERATURE CITED

- (1) Kratz, F., Müller, I. A., Ryppa, C., and Warnecke, A. (2008) Prodrug strategies in anticancer chemotherapy. *ChemMedChem* 3, 20–53.
- (2) Panchal, R. G. (1998) Novel therapeutic strategies to selectively kill cancer cells. *Biochem. Pharmacol.* 55, 247–252.
- (3) Zangemeister-Wittke, U. (2005) Antibodies for targeted cancer therapy - technical aspects and clinical perspectives. *Pathobiology* 72, 279–286.
- (4) Monoclonal antibodies conjugated with radioisotopes do not have to release their payload at the target site and need thus completely different linkers than mAb conjugated with cytotoxins. They are not reviewed in this article. For a review, see Wu, A. M., and Senter, P. D. (2005) Arming antibodies: Prospects and challenges for immunoconjugates. *Nat. Biotechnol.* 23, 1137–1146.
- (5) Ricart, A. D., and Tolcher, A. W. (2007) Technology insight: Cytotoxic drug immunoconjugates for cancer therapy. *Nat. Clin. Pract. Oncol.* 4, 245–255.
- (6) Carter, P. J., and Senter, P. D. (2008) Antibody-drug conjugates for cancer therapy. *Cancer J.* 14, 154–169.
- (7) Rohrer, T. (2008) New weapons for the oncology arsenal. *Chem. Rundschau* 9, 26–28.
- (8) Hamblett, K. J., Senter, P. D., Chace, D. F., Sun, M. M. C., Lenox, J., Cervený, C. G., Kissler, K. M., Bernhardt, S. X., Kopcha, A. K., Zabinski, R. F., Meyer, D. L., and Francisco, J. A. (2004) Effects of drug loading on the antitumor activity of

- a monoclonal antibody drug conjugate. *Clin. Cancer Res.* 10, 7063–7070.
- (9) McDonagh, C. F., Turcott, E., Westendorf, L., Webster, J. B., Alley, S. C., Kim, K., Andreyka, J., Stone, I., Hamblett, K. J., Francisco, J. A., and Carter, P. (2006) Engineered antibody-drug conjugates with defined sites and stoichiometries of drug attachment. *Prot. Eng. Des. Sel.* 19, 299–307.
- (10) Trail, P. A., Willner, D., Lasch, S. J., Henderson, A. J., Hofstead, S., Casazza, A. M., Firestone, R. A., Hellström, I., and Hellström, K. E. (1993) Cure of xenografted human carcinomas by BR96-doxorubicin immunoconjugates. *Science* 261, 212–215.
- (11) Dubowchik, G. M., Firestone, R. A., Padilla, L., Willner, D., Hofstead, S. J., Mosure, K., Knipe, J. O., Lasch, S. J., and Trail, P. A. (2002) Cathepsin B-labile dipeptide linkers for lysosomal release of doxorubicin from internalizing immunoconjugates: Model studies of enzymatic drug release and antigen specific *in vitro* anticancer activity. *Bioconjugate Chem.* 13, 855–869.
- (12) Hamann, P. R., Hinman, L. M., Beyer, C. F., Lindh, D., Upeslakis, J., Flowers, D. A., and Bernstein, I. (2002) An anti-CD33 antibody-calicheamicin conjugate for treatment of acute myeloid leukaemia. Choice of linker. *Bioconjugate Chem.* 13, 40–46.
- (13) Hamann, P. R., Hinman, L. M., Hollander, I., Beyer, C. F., Lindh, D., Holcomb, R., Hallett, W., Tsou, H.-R., Upeslakis, J., Shochat, D., Mountain, A., Flowers, D. A., and Bernstein, I. (2002) Gemtuzumab ozogamicin, a potent and selective anti-CD33 antibody-calicheamicin conjugate for treatment of acute myeloid leukemia. *Bioconjugate Chem.* 13, 47–58.
- (14) Hollander, I., Kunz, A., and Hamann, P. R. (2008) Selection of reaction additives used in the preparation of monomeric antibody-calicheamicin conjugates. *Bioconjugate Chem.* 19, 358–361.
- (15) van der Velden, V. H. J., te Marvelde, J. G., Hoogeveen, P. G., Bernstein, I. D., Houtsmuller, A. B., Berger, M. S., and van Dongen, J. J. M. (2001) Targeting of the CD33-calicheamicin immunoconjugate Mylotarg (CMA-676) in acute myeloid leukemia: *in vivo* and *in vitro* saturation and internalization by leukemic and normal myeloid cells. *Blood* 97, 3197–3204.
- (16) Hamann, P. R., Hinman, L. M., Beyer, C. F., Lindh, D., Upeslakis, J., Shochat, D., and Mountain, A. (2005) A calicheamicin conjugate with a fully humanized anti-MUC1 antibody shows potent antitumor effects in breast and ovarian tumor xenografts. *Bioconjugate Chem.* 16, 354–360.
- (17) DiJoseph, J. F., Dougher, M. M., Kalyandrug, L. B., Armellino, D. C., Boghaert, E. R., Hamann, P. R., Moran, J. K., and Damle, N. K. (2006) Antitumor efficacy of a combination of CMC-544 (inotuzumab ozogamicin), a CD22-targeted cytotoxic immunoconjugate of calicheamicin, and rituximab against non-Hodgkin's B-cell lymphoma. *Clin. Cancer Res.* 12, 242–249.
- (18) DiJoseph, J. F., Khandke, K., Dougher, M. M., Evans, D. Y., Armellino, D. C., Hamann, P. R., and Damle, N. K. (2008) CMC-544 (inotuzumab ozogamicin): A CD22-targeted immunoconjugate of calicheamicin. *Hematology Meeting Reports* 5, 74–77.
- (19) Ojima, I., Geng, X., Wu, X., Qu, C., Borella, C. P., Xie, H., Wilhelm, S. D., Leece, B. A., Bartle, L. M., Goldmacher, V. S., and Chari, R. V. J. (2002) Tumor-specific novel taxoid-monoclonal antibody conjugates. *J. Med. Chem.* 45, 5620–5623.
- (20) Ranson, M., and Sliwkowski, M. X. (2002) Perspectives on anti-HER monoclonal antibodies. *Oncology* 63 (suppl. 1), 17–24.
- (21) Widdison, W. C., Wilhelm, S. D., Cavanagh, E. E., Whiteman, K. R., Leece, B. A., Kovtun, Y., Goldmacher, V. S., Xie, H., Steeves, R. M., Lutz, R. J., Zhao, R., Wang, L., Blättler, W. A., and Chari, R. V. J. (2006) Semisynthetic maytansine analogues for the targeted treatment of cancer. *J. Med. Chem.* 49, 4392–4408.
- (22) Erickson, H. K., Park, P. U., Widdison, W. C., Kovtun, Y. V., Garrett, L. M., Hoffman, K., Lutz, R. J., Goldmacher, V. S., and Blättler, W. A. (2006) Antibody-Maytansinoid conjugates are activated in targeted cancer cells by lysosomal degradation and linker-dependent intracellular processing. *Cancer Res.* 66, 4426–4433.
- (23) Kovtun, Y. V., Audette, C. A., Ye, Y., Xie, H., Ruberti, M. F., Phinney, S. J., Leece, B. A., Chittenden, T., Blättler, W. A., and Goldmacher, V. S. (2006) Antibody-drug conjugates designed to eradicate tumors with homogeneous and heterogeneous expression of the target antigen. *Cancer Res.* 66, 3214–3221.
- (24) Xie, H., Audette, C., Hoffee, M., Lambert, J. M., and Blättler, W. A. (2004) Pharmacokinetics and biodistribution of the antitumor immunoconjugate, cantuzumab mertansine (huC242-DM1), and its two components in mice. *J. Pharmacol. Exp. Ther.* 308, 1073–1082.
- (25) Kobliński, J. E., Ahram, M., and Sloane, B. F. (2000) Unraveling the role of proteases in cancer. *Clin. Chim. Acta* 291, 113–135.
- (26) Doronina, S. O., Toki, B. E., Torgov, M. Y., Mendelsohn, B. A., Cervený, C. G., Chace, D. F., DeBlanc, R. L., Gearing, R. P., Bovee, T. D., Siegall, C. B., Francisco, J. A., Wahl, A. F., Meyer, D. L., and Senter, P. D. (2003) Development of potent monoclonal antibody auristatin conjugates for cancer therapy. *Nat. Biotechnol.* 21, 778–784.
- (27) Toki, B. E., Cervený, C. G., Wahl, A. F., and Senter, P. D. (2002) Protease-mediated fragmentation of *p*-amidobenzyl ethers: A new strategy for the activation of anticancer prodrugs. *J. Org. Chem.* 67, 1866–1872.
- (28) Sanderson, R. J., Hering, M. A., James, S. F., Sun, M. M. C., Doronina, S. O., Siadak, A. W., Senter, P. D., and Wahl, A. F. (2005) *In vivo* drug-linker stability of an anti-CD30 dipeptide-linked auristatin immunoconjugate. *Clin. Cancer Res.* 11, 843–852.
- (29) Francisco, J. A., Cervený, C. G., Meyer, D. L., Mixan, B. J., Klussman, K., Chace, D. F., Rejniak, S. X., Gordon, K. A., DeBlanc, R., Toki, B. E., Law, C.-L., Doronina, S. O., Siegall, C. B., Senter, P. D., and Wahl, A. F. (2003) cAC10-vcMMAE, an anti-CD30-monomethyl auristatin E conjugate with potent and selective antitumor activity. *Blood* 102, 1458–1465.
- (30) Bartlett, N., Forero-Torres, A., Rosenblatt, J., Fanale, M., Horning, S. J., Thompson, S., Sievers, E. L., and Kennedy, D. A. (2009) Complete remissions with weekly dosing of SGN-35, a novel antibody-drug conjugate (ADC) targeting CD30, in phase I dose-escalation study in patients with relapsed or refractory Hodgkin lymphoma (HL) or systemic anaplastic large cell lymphoma (sALCL). *J. Clin. Oncol.* 27, 8500.
- (31) Doronina, S. O., Bovee, T. D., Meyer, D. W., Miyamoto, J. B., Anderson, M. E., Morris-Tilden, C. A., and Senter, P. D. (2008) Novel peptide linkers for highly potent antibody-auristatin conjugate. *Bioconjugate Chem.* 19, 1960–1963.
- (32) Jeffrey, S. C., Nguyen, M. T., Andreyka, J. B., Meyer, D. L., Doronina, S. O., and Senter, P. D. (2006) Dipeptide-based highly potent doxorubicin antibody conjugates. *Bioorg. Med. Chem. Lett.* 16, 358–362.
- (33) Boger, D. L., Boyce, C. W., Garbaccio, R. M., Searcey, M., and Jin, Q. (1999) CBI prodrug analogs of CC-1065 and the duocarmycins. *Synthesis* 1505–1509, and references cited therein.
- (34) Nagamura, S., Kanda, Y., Kobayashi, E., Gomi, K., and Saito, H. (1995) Synthesis and antitumor activity of duocarmycin derivatives. *Chem. Pharm. Bull.* 43, 1530–1535.
- (35) Baird, R., and Winstein, S. (1963) Neighboring carbon and hydrogen. LI. Dienones from $Ar_1\theta-3$ participation. Isolation and behavior of spiro(2,5)octa-1,4-diene-3-one. *J. Am. Chem. Soc.* 85, 567–578.
- (36) Wang, Y., Li, L., Tian, Z., Jiang, W., and Larrick, J. W. (2006) Synthesis and antitumor activity of CBI-bearing ester and carbamate prodrugs of CC-1065 analogue. *Bioorg. Med. Chem.* 14, 7854–7861.
- (37) Chen, L., Gangwar, S., Guerlavais, V., Lonberg, N., Zhang, Q. Chemical linkers with single amino acids and conjugates thereof, international patent application, WO 2008/103693 A2.
- (38) Longberg, N. (2008) Medarex '2008 R&D Day', New York City.

- (39) Tietze, L. F., Feuerstein, T., Fecher, A., Haunert, F., Panknin, O., Borchers, U., Schuberth, I., and Alves, F. (2002) Proof of principle in the selective treatment of cancer by antibody-directed enzyme prodrug therapy: The development of a highly potent prodrug. *Angew. Chem., Int. Ed.* **41**, 759–761.
- (40) Tietze, L. F., Herzig, T., Feuerstein, T., and Schuberth, I. (2002) Synthesis and biological evaluation of novel analogues and prodrugs of the cytotoxic antibiotic CC-1065 for selective cancer therapy. *Eur. J. Org. Chem.* 1634–1645.
- (41) Tietze, L. F., Major, F., Schuberth, I., Spiegl, D. A., Krewer, B., Maksimenka, K., Bringmann, G., and Magull, J. (2007) Selective treatment of cancer: Synthesis, biological evaluation and structural elucidation of novel analogues of the antibiotic CC-1065 and the duocarmycins. *Chem.—Eur. J.* **13**, 4396–4409.
- (42) Beusker, P. H., de Groot, F. M. H., Tietze, L. F., Major, F., Joosten, J. A. F., Spijker, H. J. Water-soluble CC-1065 analogs and their conjugates, international patent application, WO 2007/089149 A2.
- (43) Jeffrey, S. C., Torgov, M. Y., Andreyka, J. B., Boddington, L., Cervený, C. G., Denny, W. A., Gordon, K. A., Gustin, D., Haugen, J., Kline, T., Nguyen, M. T., and Senter, P. D. (2005) Design, synthesis, and in vitro evaluation of dipeptide-based antibody minor groove binder conjugates. *J. Med. Chem.* **48**, 1344–1358.
- (44) Jeffrey, S. C., Nguyen, M. T., Moser, R. F., Meyer, D. L., Miyamoto, J. B., and Senter, P. D. (2007) Minor groove binder antibody conjugates employing a water soluble β -glucuronide linker. *Bioorg. Med. Chem. Lett.* **17**, 2278–2280.
- (45) Burke, P. J., Senter, P. D., Meyer, D. W., Miyamoto, J. B., Anderson, M., Toki, B. E., Manikumar, G., Wani, M. C., Kroll, D. J., and Jeffrey, S. C. (2009) Design, synthesis, and biological evaluation of antibody-drug conjugates comprised of potent Camptothecin analogues. *Bioconjugate Chem.* **20**, 1242–1250.
- (46) Chari, R. V. J. (2008) Targeted cancer therapy: Conferring specificity to cytotoxic drugs. *Acc. Chem. Res.* **41**, 98–107.
- (47) Polson, A. G., Calemme-Fenau, J., Chan, P., Chang, W., Christensen, E., Clark, S., de Sauvage, F. J., Eaton, D., Elkins, K., Elliott, J. M., Frantz, G., Fuji, R. N., Gray, A., Harden, K., Ingle, G. S., Kljavin, N. M., Koeppen, H., Nelson, C., Prabhu, S., Raab, H., Ross, S., Stephan, J.-P., Scales, S. J., Spencer, S. D., Vandlen, R., Wranik, B., Yu, S.-F., Zheng, B., and Ebens, A. (2009) Antibody-drug conjugates for the treatment of non-Hodgkin's lymphoma: Target and linker-drug selection. *Cancer Res.* **69**, 2358–2364.
- (48) Vogel, C. L., Burris, H. A., Limentani, S., Borson, R., O'Shaughnessy, J., Vukelja, S., Agresta, S., Klencke, B., Birkner, M., and Rugo, H. (2009) A phase II study of trastuzumab-DM1 (T-DM1), a HER2 antibody-drug conjugate (ADC), in patients (pts) with HER2+ metastatic breast cancer (MBC): Final results. *J. Clin. Oncol.* **27**:15s (suppl; abstr 1017).
- (49) Doronina, S. O., Mendelsohn, B. A., Bovee, T. D., Cervený, C. G., Alley, S. C., Meyer, D. L., Oflazoglu, E., Toki, B. E., Sanderson, R. J., Zabinski, R. F., Wahl, A. F., and Senter, P. D. (2006) Enhanced activity of monomethylauristatin F through monoclonal antibody delivery: Effects of linker technology on efficacy and toxicity. *Bioconjugate Chem.* **17**, 114–124.
- (50) Alley, S. C., Benjamin, D. R., Jeffrey, S. C., Okeley, N. M., Meyer, D. L., Sanderson, R. J., and Senter, P. D. (2008) Contribution of linker stability to the activities of anticancer immunoconjugates. *Bioconjugate Chem.* **19**, 759–765.

BC9002019

Preclinical evaluation of MORAb-009, a chimeric antibody targeting tumor-associated mesothelin

Raffit Hassan^{1*}, Wolfgang Ebel^{2*}, Eric L. Routhier², Rina Patel², J. Bradford Kline², Jingli Zhang¹, Qimin Chao², Sara Jacob², Howard Turchin², Lester Gibbs², Martin D. Phillips², Shiyama Mudali³, Christine Iacobuzio-Donahue³, Elizabeth M. Jaffee⁴, Maria Moreno⁵, Ira Pastan¹, Philip M. Sass², Nicholas C. Nicolaides² and Luigi Grasso²

¹Laboratory of Molecular Biology, National Cancer Institute, Bethesda, MD, USA

²Morphotek Inc., Exton, PA, USA

³Drug Development Program, The Sol Goldman Pancreatic Cancer Research Center, Johns Hopkins University, Baltimore, MD, USA

⁴Department of Oncology, The Sol Goldman Pancreatic Cancer Research Center, Johns Hopkins University, Baltimore, MD, USA

⁵Obstetrics and Gynecology, VU Medical Centre, Amsterdam, The Netherlands

*These authors contributed equally to this work

Communicated by: V Cerundolo

Novel therapeutic agents that are safe and effective are needed for the treatment of pancreatic, ovarian, lung adenocarcinomas and mesotheliomas. Mesothelin is a glycosyl-phosphatidyl inositol (GPI)-linked membrane protein of 40 kDa over-expressed in all pancreatic adenocarcinoma and mesothelioma, in >70% of ovarian adenocarcinoma, and in non-small cell lung and colorectal cancers. The biological functions of mesothelin are not known, although it appears to be involved in cell adhesion via its interaction with MUC16. We have recently developed MORAb-009, a mouse-human chimeric IgG1κ monoclonal antibody with an affinity of 1.5 nM for human mesothelin. Here we provide evidence that MORAb-009 prevents adhesion of mesothelin-bearing tumor cells to MUC16 positive cells and can elicit cell-mediated cytotoxicity on mesothelin-bearing tumor cells. Treatment that included MORAb-009 in combination with chemotherapy led to a marked reduction in tumor growth of mesothelin-expressing tumors in nude mice compared to chemotherapy or MORAb-009 treatment alone. No adverse effects of MORAb-009 were noted during toxicology studies conducted in non-human primates. The preclinical data obtained from our studies warrants pursuing clinical testing of MORAb-009. We have in fact initiated a Phase I clinical study enrolling patients with mesothelin-positive pancreatic, mesothelioma, non-small cell lung and ovarian cancers.

Keywords: preclinical drug evaluation, monoclonal antibody, mesothelin, antibody-dependent cellular cytotoxicity

Introduction

Mesothelin is a glycosyl-phosphatidyl inositol-linked membrane glycoprotein expressed in a variety of cancers (1, 2). Immunohistochemistry studies have shown that mesothelin is over-expressed in virtually all pancreatic ductal adenocarcinomas and mesotheliomas (3-5), a high percentage of epithelial ovarian cancers (including fallopian tube and primary peritoneal malignancies), as well as non-small cell carcinomas of the lung (6-10). Expression in normal tissues is limited to mesothelial cells lining the pleura, pericardium and peritoneum (6).

Since mesothelin has recently been reported to bind to CA125/MUC16, one possible biological role of this membrane protein

may be linked to heterotypic cell adhesion, which may facilitate the metastatic spread of mesothelin-bearing cancer cells (11). Notwithstanding, the biologic functions of mesothelin remain to be fully defined. Analysis of the mesothelin amino acid sequence yielded no strong homologies to known protein functional domains, other than a C-terminal GPI-anchor motif (2). Moreover, mesothelin does not appear to be required for either normal development or reproduction since there were no apparent abnormalities in mesothelin knockout mice (12).

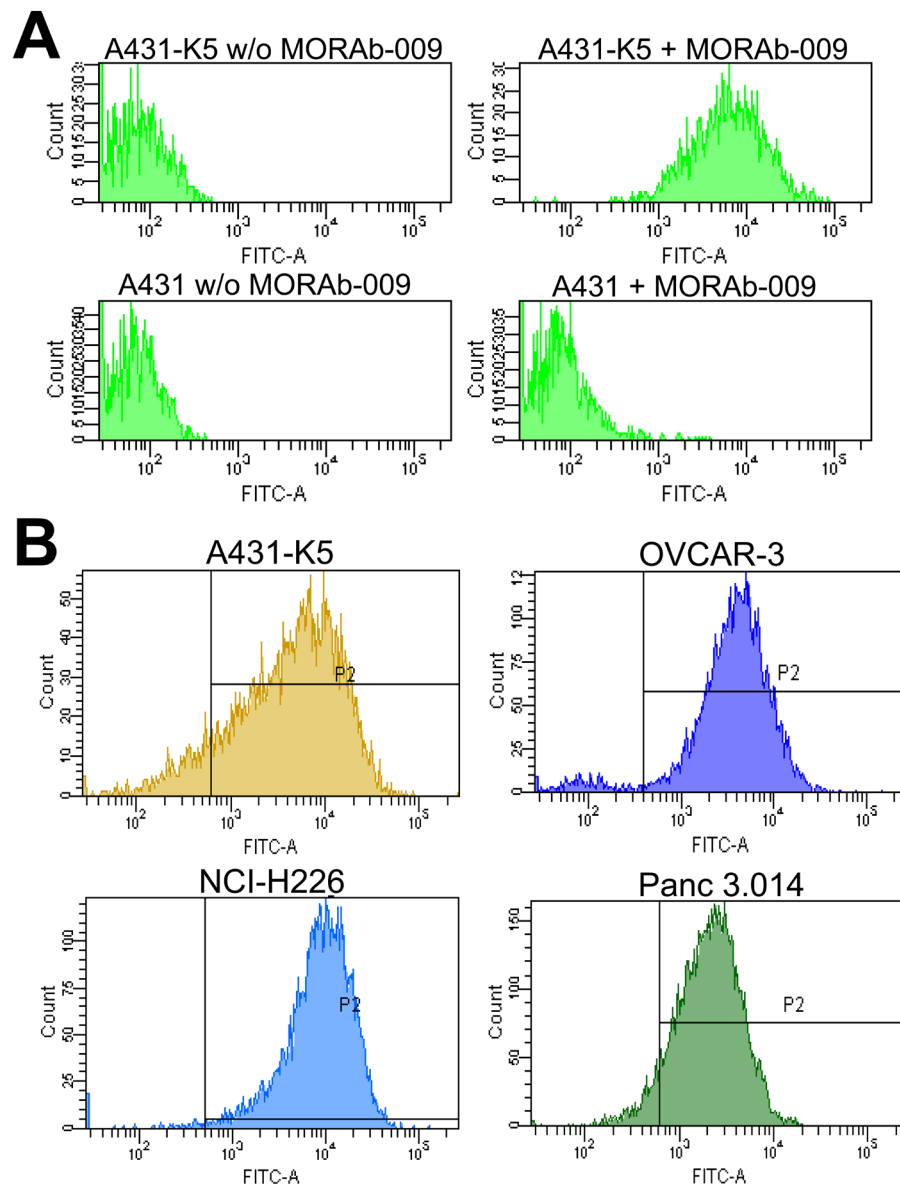
Two lines of clinical evidence suggest that mesothelin may be an important target for the treatment of mesothelin-bearing tumors. First, tumor responses were observed in a Phase I trial of the anti-mesothelin immunotoxin, SS1P, which included patients with mesotheliomas, ovarian and pancreatic cancer (13). In a different clinical trial, induction of cytotoxic T cell responses to mesothelin epitopes were exclusively obtained in three patients vaccinated with autologous mesothelin-positive tumor cells who had shown delayed-type hypersensitivity after vaccination (14).

Novel therapeutic agents that are safe and effective are needed for the treatment of pancreatic, ovarian, mesothelioma, and lung cancers. Over the years, monoclonal antibodies have proven to be well tolerated, disease antigen-specific, and able to deliver clinical benefits to patients with conditions that include chronic inflammation, lymphomas, and metastatic solid tumors (15). Here we report the preclinical evaluation of MORAb-009, a chimeric monoclonal antibody antagonizing human mesothelin to determine its suitability as an anti-tumor agent. MORAb-009 was able to mediate biological responses *in vitro*, including inhibition of mesothelin-dependent cell adhesion, antibody-dependent cellular cytotoxicity (ADCC), as well as the reduction of tumor growth in mice implanted with human cancer cells, while exhibiting no adverse effects in non-human primates.

Results

Generation of MORAb-009

The mouse antibody precursor of MORAb-009 was first isolated by panning a phage display library made from splenic mRNA of a mouse immunized with mesothelin cDNA on mesothelin-positive cells (16). The affinity of this mouse single-

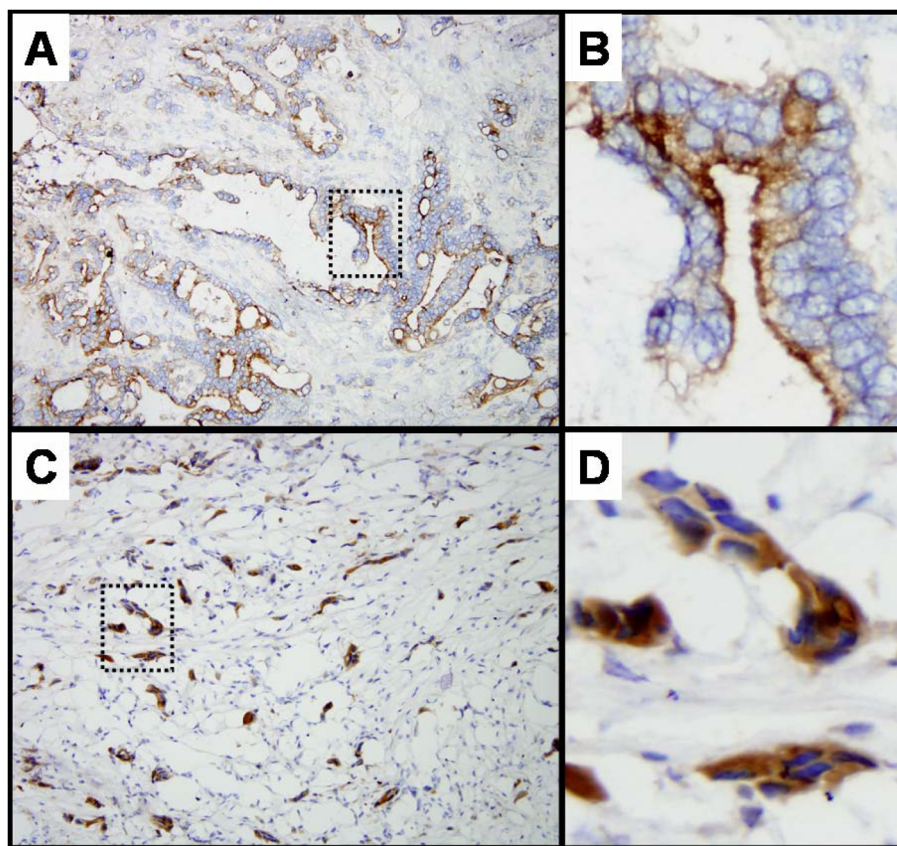
Figure 1

FACS analyses of tumor cells stained with MORAb-009. (A) Parental A431 cells, as well as cells expressing mesothelin (A431-K5), were reacted with FITC-labeled anti-human IgG antibody, with or without MORAb-009. Positive staining was observed only in the presence of MORAb-009 in A431-K5 cells, demonstrating specificity of the immune staining and lack of reactivity of MORAb-009 with other unrelated membrane proteins. (B) Tumor cells were reacted with FITC-labeled anti-human IgG antibody alone to determine background staining (not shown) and to create gate P2 to capture fluorescent signal above background. MORAb-009 was able to bind mesothelin expressed by ovarian (OVCAR-3), mesothelioma (NCI-H226), and pancreatic (Panc 3.014) cancer cells.

chain anti-mesothelin antibody was further optimized by re-engineering its variable regions to derive SS1(scFv) (17). Subsequently, we have cloned the heavy and light chain variable regions of SS1(scFv) and grafted them in frame with human IgG1 and kappa constant regions, respectively, yielding the genes coding for a chimeric (mouse/human) antibody designated MORAb-009 showing an affinity (K_D) of 1.5 nM. When aligned to its closest human germline immunoglobulin match, MORAb-009 exhibits a high amino acid sequence identity of 82.6% (excluding the complementarity determining regions), compared to a typical 90% identity of a humanized antibody.

MORAb-009 binds to cell surface mesothelin expressed by tumor cell lines

To test the binding specificity of MORAb-009, we conducted FACS analyses using the mesothelin negative and positive cells, A431 and A431-K5, respectively. A431-K5 cells were derived from human epidermoid carcinoma cells (A431) ectopically expressing membrane-bound human mesothelin (6). Surface binding by MORAb-009 was only observed on A431-K5 cells (Figure 1A, top right panel), indicating that MORAb-009 specifically bound mesothelin but not other membrane proteins expressed by these cancer cells. Mesothelin is frequently

Figure 2

Immunohistochemical staining of human pancreatic tumor tissues. Frozen sections of ductal pancreatic adenocarcinomas (A and C) were stained with MORAb-009. Membranous staining at the luminal and apical surface of neoplastic cells was detected. (B and D) Five-fold enlargement of dotted areas in A and C. Shown are clusters of neoplastic cells where approximately 75% of the cells stained positive with MORAb-009.

expressed in pancreatic, mesothelioma and ovarian cancers. We therefore tested whether MORAb-009 could recognize mesothelin on the cell surface of ovarian (OVCAR-3), mesothelioma (NCI-H226), and pancreatic (Panc 3.014) cancer cell lines known to express mesothelin. Significant MORAb-009 binding could be detected in all cell lines tested (Figure 1B) suggesting that this antibody is suitable for targeting tumor-associated mesothelin.

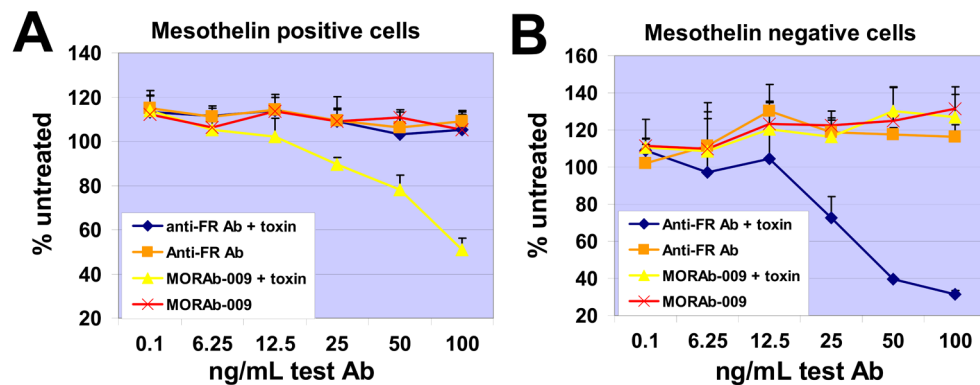
MORAb-009 targets tumor-associated mesothelin

Immunohistochemistry studies supported the ability of MORAb-009 to target mesothelin protein expressed in human pancreatic tumors. We detected membranous staining at the luminal and apical surface of neoplastic cells, although some cytoplasmic staining was also observed (Figure 2). Of the ten frozen pancreatic ductal adenocarcinomas (primary and metastatic disease) studied, all were found to be positive for MORAb-009 staining, with 50% of pancreatic cancer specimens showing strong positive labeling (3+) of at least 75% of the neoplastic cells and the remaining specimens exhibiting a less intensive staining (data not shown). This frequency of mesothelin staining seen with MORAb-009 in pancreatic cancer specimens matched that we have observed using the murine 5B2 antibody (data not shown) and is in line with previous immunohistochemistry studies using the same murine antibody (3). No relationship between tumor stage (primary versus

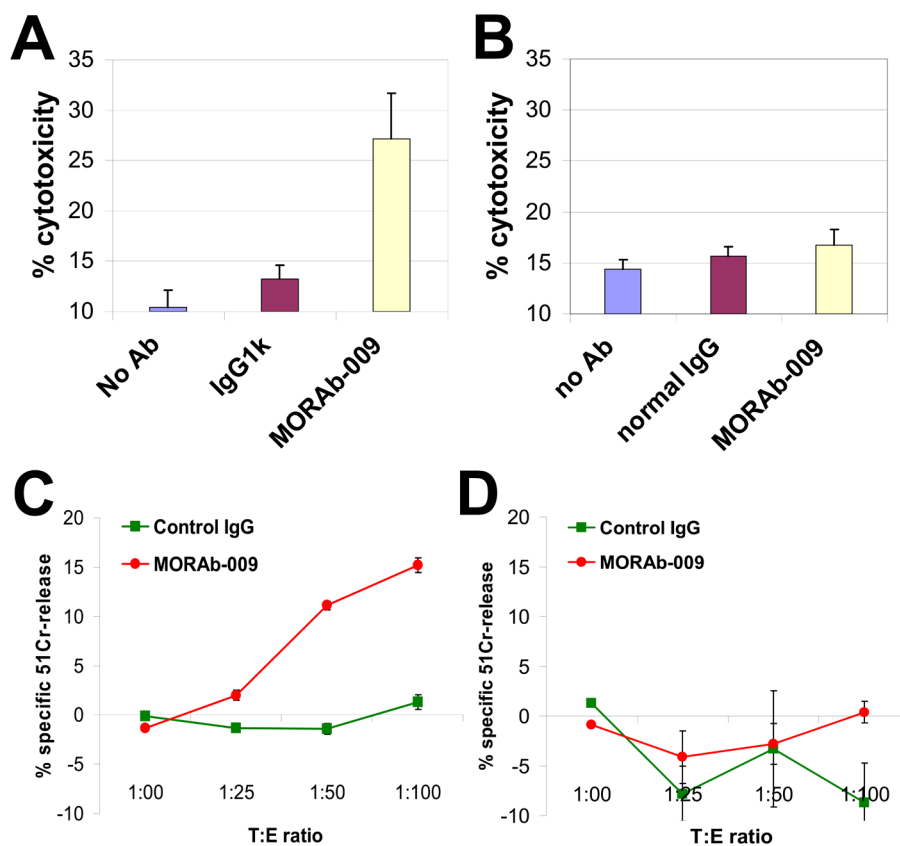
metastasis) and staining intensity was found, although we observed that well differentiated carcinomas tended to stain more robustly than poorly differentiated carcinomas.

Internalization of MORAb-009 upon binding to cell surface mesothelin

Saporin is a toxin that inhibits protein translation causing cell death only if it is internalized by cells and can be used to determine receptor internalization (18). To address whether MORAb-009 internalizes after binding cell surface mesothelin, we reacted MORAb-009 with an anti-human IgG conjugated with saporin, treated NCI-H226 cells (mesothelin positive) or IGROV-1 cells (mesothelin negative) with this complex, and measured cell death. The MORAb-009-saporin complex, but not MORAb-009 alone, caused significant death of NCI-H226 cells (Figure 3A). A control antibody specific to folate receptor α (FR α), which is not expressed in NCI-H226 cells, expectedly had no effect on cell viability whether alone or complexed with saporin-conjugated anti-human IgG. Neither MORAb-009-saporin complex nor MORAb-009 alone caused significant death of mesothelin negative, FR α positive IGROV-1 cells (Figure 3B). These results suggest that MORAb-009 can internalize after binding mesothelin on the cell surface.

Figure 3

Internalization of MORAb-009-mesothelin complex. (A) Mesothelin positive, FR α -negative cells were reacted with MORAb-009 alone, MORAb-009 complexed with saporin-conjugated anti-human mAb (toxin), anti-FR α mAb alone, or anti-FR α mAb complexed with toxin. Cell killing, and thus internalization, of MORAb-009-mesothelin complex was only seen with MORAb-009 complexed with toxin. (B) Same as in A using mesothelin-negative, FR α -positive cells. Internalization was only seen with anti-FR α mAb complexed with toxin.

Figure 4

MORAb-009 induces ADCC. (A) OVCAR-3 cells were used as target cells and reacted with 10 μ g/mL of MORAb-009 or control IgG in the presence of human PBMCs at a ratio of 1:25. ADCC activity was measured by LDH release and only noted with MORAb-009 treatment. (B) Same as in A, whereby target cells (Panc 3.27) express low or no mesothelin. (C) NCI-H226 cells were used as target cells and reacted with 10 μ g/mL of MORAb-009 or control IgG in the presence of human PBMCs at different ratios. ADCC activity was measured by 51 Cr release and only noted with MORAb-009 treatment. (D) Same as in C, whereby target cells (Panc 10.05) express no or low levels of mesothelin.

MORAb-009 can exert antibody-dependent cellular cytotoxicity

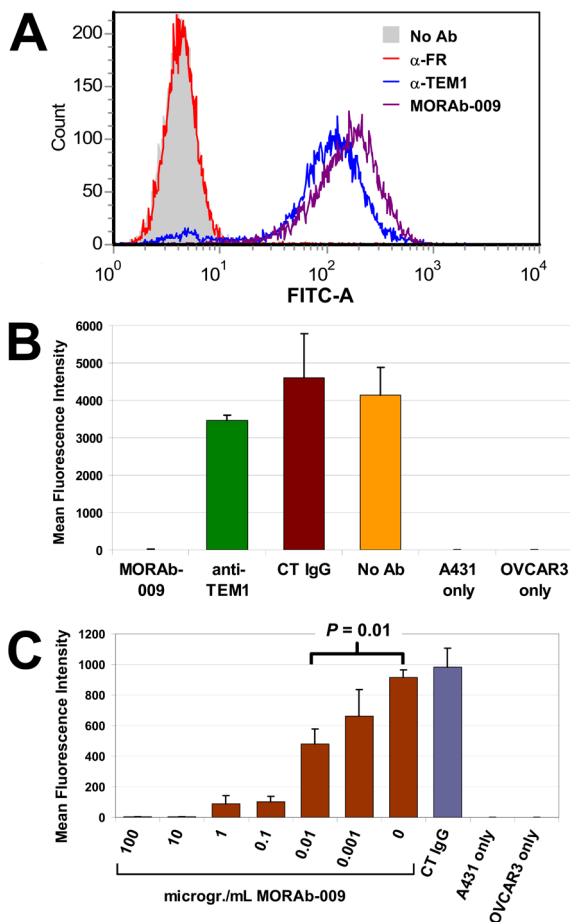
Antibody opsonization of tumor cells followed by the recruitment of immune killer cells eliciting cell-mediated cytotoxicity (ADCC) is thought to be an important mechanism of tumor cell cytotoxicity *in vivo*. To assess the ability of MORAb-009 to elicit this effector function, we used human cancer cell lines as targets for ADCC. In one strategy, measurement of lactate dehydrogenase (LDH) released by lysed cells was used to determine the extent of tumor cell killing using a constant target to effector ratio of 1:25. MORAb-009 exerted significant ADCC activity against OVCAR-3 cells (Figure 4A) but not on mesothelin negative pancreatic cancer cells, Panc 3.27 (Figure 4B). MORAb-009 also elicited significant ADCC activity on pancreatic cancer cells, Panc 3.014 (data not shown). We also used ^{51}Cr release to measure cell cytotoxicity and tested various target to effector ratios. MORAb-009 again mediated ADCC activity on mesothelin-expressing mesothelioma cells NCI-H226 (Figure 4C) but had no effect on human pancreatic cancer cells, Panc 10.05 (Figure 4D), which express low or undetectable mesothelin levels (data not shown). Maximum effect was seen at a target to effector ratio of 1:100. We did not measure significant complement-mediated cytotoxicity (CDC) by MORAb-009 (data not shown). Since our preliminary mapping identified an epitope for MORAb-009 at the amino terminus of mesothelin, one untested hypothesis is that mesothelin-bound MORAb-009 may be too far from the cell surface for the complement membrane attack complex to be effective.

CA125/MUC16 and mesothelin-dependent cell adhesion is blocked by MORAb-009

CA125/MUC16 has been identified as a ligand for mesothelin (11). CA125/MUC16 and mesothelin are both expressed in the peritoneal mesothelium as well as in some ovarian cancer cells and are believed to facilitate metastasis of cancer cells to the peritoneum by promoting cell attachment (19). In our preliminary studies, A431-K5 cells, but not parental A431 cells, could bind to CA125/MUC16 positive cells, demonstrating that this heterotypic cell interaction depends on mesothelin expression (data not shown). To explore the possibility that MORAb-009 interferes with mesothelin-CA125/MUC16 interaction, we established A431 cells (A431-K5-TEM) that expressed mesothelin as well as another surface receptor, endosialin/TEM1, but are negative for FR α . Figure 5A shows FACS analysis of A431-K5-TEM cells that exhibited positive staining after being reacted with either MORAb-009 or an anti-endosialin/TEM1 antibody. Expectedly, A431-K5-TEM cells did not stain with an anti-FR α antibody, demonstrating specificity of binding. We then measured adhesion of fluorescently labeled A431-K5-TEM cells onto CA125/MUC16 positive OVCAR-3 cells. Figure 5B outlines the results of this study. In the absence of OVCAR-3 cells, no adhesion of A431-K5-TEM cells to the uncoated surface of the microplate could be observed. Significant heterotypic cell binding was seen on a monolayer of OVCAR-3 cells in the absence of antibody treatment or with control IgG. In contrast, MORAb-009 completely abolished A431-K5-TEM cell adhesion onto OVCAR-3 cells. Although anti-endosialin/TEM1 antibody could bind A431-K5-TEM cells as strongly as MORAb-009 (Figure 5A), it did not inhibit cell-cell interaction, suggesting that MORAb-009-mediated inhibition of cell adhesion was due to specific disruption of mesothelin-CA125/MUC16 interaction rather than steric hindrance at the cell-cell interface. The ED $_{90}$ of this activity was

estimated around 100 ng/mL of MORAb-009 and a statistically significant inhibition was detected with concentrations as low as 10 ng/mL (Figure 5C).

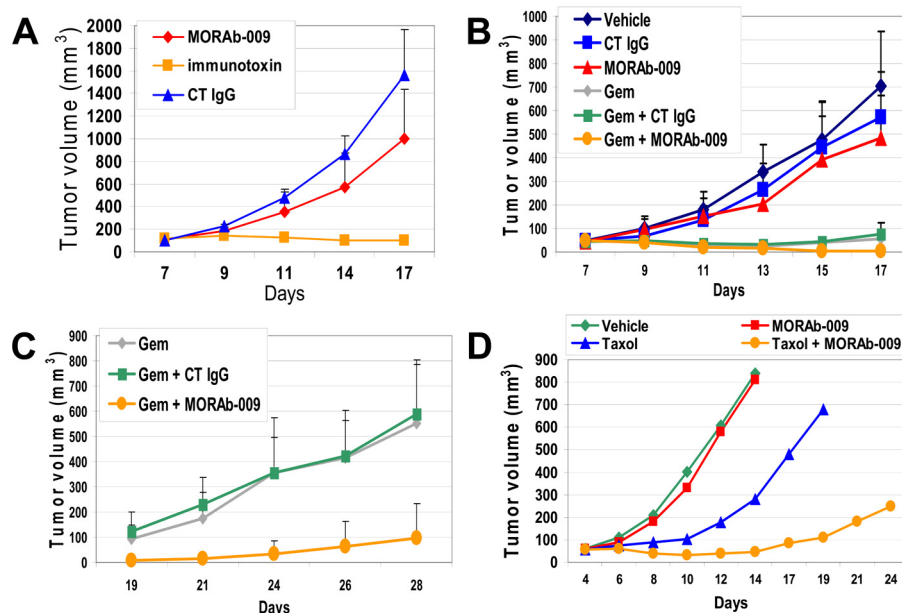
Figure 5



MORAb-009 blocks CA125/MUC16-mesothelin interaction. (A) FACS analysis of A431-K5-TEM cells reacted with no mAb, anti-FR α mAb, anti-TEM1 mAb, or MORAb-009. Equivalent positive staining was associated with the expression of cognate proteins (TEM-1 and mesothelin) but not with FR α , which is not expressed in these cells. (B) Fluorescently-labeled A431-K5-TEM cells were reacted with no mAb, anti-FR α mAb (CT IgG), anti-TEM1 mAb, or MORAb-009, and seeded on a monolayer of CA125/MUC16 positive OVCAR-3 cells (first four bars). Cell adhesion was completely blocked by MORAb-009 compared to the other treatments. (C) Same as in B, whereby MORAb-009 concentration ranged from 1 ng/mL to 100 μ g/mL. Blockade of CA125/MUC16-mesothelin interaction was seen with concentrations as low as 10 ng/mL.

MORAb-009 enhances the anti-tumor effects of chemotherapy *in vivo*

The *in vivo* anti-tumor effect of MORAb-009 in combination with chemotherapy was evaluated in immunodeficient mice bearing A431-K5 tumor xenografts. The number of receptor sites in these cells is comparable to that of other tumor cells endogenously expressing mesothelin (Figure 1B) and their implantation in mice consistently results in aggressive tumor

Figure 6

Effect of MORAb-009 on tumor growth. (A) A431-K5 cells were inoculated in the flank of nude mice to establish tumors of approximately 50 mm³ in size. On day 7, mice were treated with the control IgG1 Rituximab (CT IgG, 50 mg/kg), MORAb-009 (50 mg/kg), or mesothelin-specific immunotoxin SS1(scFv) (immunotoxin, 0.2 mg/kg). Average tumor size for each treatment group was calculated on day 7-17. (B and C) A431-K5 cells were inoculated as described in A. On day 7, mice were treated with vehicle, control IgG (CT IgG, 50 mg/kg), MORAb-009 (50 mg/kg), gemcitabine (Gem, 80 mg/kg), or combinations of these drugs (see Material and methods for regimens). Average tumor size for each treatment group was calculated on day 7-17 (panel B) and day 19-28 (panel C). Best anti-tumor responses were observed with gemcitabine plus MORAb-009. (D) Same model as in panels A-C, whereby mice were treated with vehicle, MORAb-009 (50 mg/kg), Taxol® (50 mg/kg), or combinations of these drugs. MORAb-009 enhanced the anti-tumor effect of Taxol®.

growth when compared to other mesothelin-positive cells. Preliminary studies using the A431-K5 tumor xenograft model showed moderate but statistically significant ($P = 0.01$) anti-tumor activity of MORAb-009 alone compared to the isotype control Rituximab, an IgG1 monoclonal antibody that targets the CD20 antigen not expressed on A431-K5 cells (Figure 6A). In this model, the mesothelin-specific immunotoxin SS1(scFv) could completely inhibit tumor growth. In subsequent studies, athymic nude mice bearing A431-K5 tumors were treated with MORAb-009 alone, gemcitabine alone (at a dose that can delay tumor growth without causing regression) or with the combination of the two agents. Seventeen days after inoculation of tumor cells, the average tumor size in mice treated with MORAb-009 alone was reduced compared to vehicle control and Rituximab alone treated mice, albeit this response was moderate and did not reach statistical significance ($P = 0.071$, Figure 6B). We observed significant tumor growth inhibition in mice treated with gemcitabine alone or in combination with MORAb-009 ($P < 0.001$), compared to control IgG (Rituximab) or MORAb-009 alone groups. Due to their tumor burden, animals in the vehicle control, Rituximab, and MORAb-009 single agent groups were sacrificed around day 17-18. The last dose of MORAb-009 or control IgG was administered on day 17, while we continued monitoring tumor volumes in the remaining groups for an additional 11 days (Figure 6C). Whereas tumors resumed vigorous growth in mice treated with gemcitabine alone, reaching an average volume of 600 mm³ by day 28, the average tumor volume in mice that also received MORAb-009 remained significantly smaller than 100 mm³ ($P = 0.001$, Figure 6C). Importantly, transient tumor remissions (tumor volumes 0-8 mm³) were only noted in the gemcitabine/

MORAb-009 treatment group (6 of the 10 mice) compared to none in the other groups, with two mice remaining tumor-free for the entire course of the study (35 days). Expectedly, the control IgG (Rituximab) had no effect on tumor growth whether administered alone or in combination with gemcitabine ($P = 0.548$). Since Taxol® is frequently used in the clinical setting as the first line therapy of mesothelin-expressing ovarian and lung adenocarcinomas, we also evaluated possible synergistic anti-tumor activity of MORAb-009 in combination with Taxol® using the above A431-K5 tumor xenograft model. As shown in Figure 6D, while treatment with MORAb-009 alone showed little tumor volume reduction and treatment with Taxol® alone only delayed tumor growth, we observed a more robust anti-tumor effect when Taxol® and MORAb-009 were used in combination. Importantly, four of the seven mice in the Taxol®/MORAb-009 combination treatment group exhibited complete tumor regression compared to none in the other groups.

MORAb-009 safety profile

Western blot analysis utilizing mesothelin-expressing tissues from rat, mouse and cynomolgus monkeys indicated lack of cross-reactivity of MORAb-009 to rodent species but significant binding to monkey tissues (data not shown). Immunohistochemistry (IHC) analysis confirmed similar staining pattern in normal tissues from human and cynomolgus macaque, with staining observed only in mesothelia. Therefore, a 23-day toxicology study with MORAb-009 was conducted in cynomolgus monkeys. This study employed relatively high doses of MORAb-009 administered over a short period to establish an initial safety profile of MORAb-009. There were no

Table 1

Mean serum pharmacokinetic parameters of MORAb-009 in male and female monkeys following intravenous infusion.

Dose (mg/kg/occasion)	Total Dose (mg/kg)	Gender	AUC _{0-529.5h} (μg.h/mL)	C _{max} (μg/mL)	T _{max} (h)
2	14	Female	41200	130	530
		Male	40400	137	482
15	105	Female	252000	752	442
		Male	354000	1170	386

abnormal clinical observations, macroscopic or microscopic findings related to the administration of MORAb-009 and the data indicated that the no observed adverse effect level (NOAEL) of MORAb-009 was greater than the seven doses of 15 mg/kg administered over 23 days.

Toxicokinetic analyses were performed and the results are summarized in Table 1. Systemic exposure of female and male monkeys to MORAb-009 increased proportionately with increasing dose. There were no gender differences at 2 mg/kg/h/dose (total dose = 14 mg/kg); however, exposures in males were greater than females at 15 mg/kg/h/dose (total dose = 105 mg/kg). Over a 7.5-fold dose range, AUC_{0-529.5h} values for females and males increased 6.12 and 8.76-fold, respectively, and C_{max} values for females and males increased 5.78 and 8.54-fold, respectively. The serum concentration levels of MORAb-009 at 30 minutes prior to and 30 minutes after the last infusion were greater than those after the second infusion, indicating a possible accumulation after repeated intravenous infusions. MORAb-009 was measurable in serum up to 1224 hours after starting the first infusion in the designated monkeys for the recovery period. MORAb-009 half-life was relatively long in designated female and male monkeys for the recovery period (341 and 285 hours, respectively). Overall, the pharmacokinetics of MORAb-009 followed the typical pattern of large biomolecules administered intravenously. The T_{max} occurred at the first time point measured after the intravenous infusion was complete (30 minutes post-infusion in this study). There was drug accumulation in the plasma following multiple infusions. The volume of distribution was initially equal to the plasma volume.

Discussion

To advance new therapeutic candidates for solid tumors expressing mesothelin, we conducted the preclinical evaluation of MORAb-009, a high affinity chimeric anti-mesothelin monoclonal antibody, and determined its suitability as an anti-tumor agent. Our results show that MORAb-009 is able to mediate biological responses *in vitro*, including inhibition of mesothelin-dependent cell adhesion and ADCC activity. In addition, MORAb-009 reduced tumor growth and enhanced the effect of chemotherapy in mice implanted with human tumor cells while exhibiting no adverse effects in non-human primates.

In order to detect possible cross-reactivity, MORAb-009 was applied to a human tissue panel that included all tissues recommended by the US Food and Drug Administration. The binding profile was essentially similar to that reported for other mesothelin-specific monoclonal antibodies, whereby reactivity to normal tissues was limited to mesothelium (data not shown). As part of a safety study, we attempted to determine the dose level at which potential toxicity may occur due to binding of MORAb-009. Since MORAb-009 does not bind to rodent tissues and its staining pattern in human and cynomolgus monkey

tissues was similar, only non-human primates were used to determine potential toxicity. The results of these studies indicated that the estimated no observed adverse effect level for MORAb-009 in cynomolgus monkeys was greater than the seven doses of 15 mg/kg administered over 23 days. The lack of toxicity observed could be explained by the limited mesothelin expression in normal tissues as evidenced by the IHC studies. Furthermore, although MORAb-009 was derived from a mouse antibody via chimerization with a human IgG1 backbone, it shares 82.6% amino acid sequence identity to a human IgG1κ. This may explain the lack of monkey anti-chimeric antibody responses observed.

Although MORAb-009 as a single agent had moderate anti-tumor activity against mesothelin-expressing tumor xenografts *in vivo*, this effect was markedly increased in combination with either gemcitabine or Taxol®. Presently, the mechanism(s) by which MORAb-009 enhances the anti-tumor effects of chemotherapeutic agents such as gemcitabine and Taxol® is unknown. One hypothesis is that binding of MORAb-009 leads to opsonization of tumor cells and their subsequent killing by cytotoxic immune cells, whereby this effect is additive or synergistic to that of chemotherapy. Mesothelin has been suggested to play a role in cell adhesion and we have shown that MORAb-009 can block mesothelin binding to CA125/MUC16 (Figure 5). Although we have no evidence that in our animal models tumor growth depends on cell adhesion, specifically on CA125/MUC16-mesothelin interaction, it is still possible that binding of MORAb-009 to mesothelin interferes with other tumor-host interactions, creating a condition whereby tumor cells are more susceptible to chemotherapy. Alternatively, gemcitabine and Taxol® can enhance MORAb-009 activity by improving tumor blood flow (20), facilitating the penetration and diffusion of MORAb-009 into the tumor. Two studies, one in mice and the other in humans, showed that Taxol® increased tumor uptake of radiolabeled antibody-interleukin 2 immunocytokine (21, 22). Regardless of the exact mechanism of the synergy between MORAb-009 and chemotherapy, this interaction could be useful in the clinic for the treatment of mesothelin-expressing cancers with MORAb-009 in combination with gemcitabine or Taxol®, two commonly used drugs for the treatment of pancreatic, ovarian, lung adenocarcinomas, and mesotheliomas.

In view of the encouraging preclinical data obtained from our studies, and to evaluate the safety profile of MORAb-009 in humans, we have initiated a Phase I clinical study enrolling patients with mesothelin-positive pancreatic, mesothelioma, non-small cell lung and ovarian cancers. Results obtained from this study will help guide the development of MORAb-009 alone or in combination with chemotherapy for the treatment of mesothelin-expressing cancers.

Abbreviations

FRα, folate receptor α

References

1. Chang K, Pastan I. Molecular cloning of mesothelin, a differentiation antigen present on mesothelium, mesotheliomas, and ovarian cancers. *Proc Natl Acad Sci U S A* 1996; **93**: 136-140. (PMID: 8552591)
2. Hassan R, Bera T, Pastan I. Mesothelin: a new target for immunotherapy. *Clin Cancer Res* 2004; **10**: 3937-3942. (PMID: 15217923)

3. Argani P, Iacobuzio-Donahue C, Ryu B, Rosty C, Goggins M, Wilentz RE, Murugesan SR, Leach SD, Jaffee E, Yeo CJ, Cameron JL, Kern SE, Hruban RH. Mesothelin is overexpressed in the vast majority of ductal adenocarcinomas of the pancreas: identification of a new pancreatic cancer marker by serial analysis of gene expression (SAGE). *Clin Cancer Res* 2001; **7**: 3862-3868. (PMID: 11751476)
4. Hassan R, Laszik ZG, Lerner M, Raffeld M, Postier R, Brackett D. Mesothelin is overexpressed in pancreaticobiliary adenocarcinomas but not in normal pancreas and chronic pancreatitis. *Am J Clin Pathol* 2005; **124**: 838-845. (PMID: 16416732)
5. Ordonez NG. Value of mesothelin immunostaining in the diagnosis of mesothelioma. *Mod Pathol* 2003; **16**: 192-197. (PMID: 12640097)
6. Chang K, Pastan I, Willingham MC. Isolation and characterization of a monoclonal antibody, K1, reactive with ovarian cancers and normal mesothelium. *Int J Cancer* 1992; **50**: 373-381. (PMID: 1735605)
7. Ordonez NG. Application of mesothelin immunostaining in tumor diagnosis. *Am J Surg Pathol* 2003; **27**: 1418-1428. (PMID: 14576474)
8. Hassan R, Kreitman RJ, Pastan I, Willingham MC. Localization of mesothelin in epithelial ovarian cancer. *Appl Immunohistochem Mol Morphol* 2005; **13**: 243-247. (PMID: 16082249)
9. Ho M, Bera TK, Willingham MC, Onda M, Hassan R, Fitzgerald D, Pastan I. Mesothelin expression in human lung cancer. *Clin Cancer Res* 2007; **13**: 1571-1575. (PMID: 17332303)
10. Miettinen M, Sarlomo-Rikala M. Expression of calretinin, thrombomodulin, keratin 5, and mesothelin in lung carcinomas of different types: an immunohistochemical analysis of 596 tumors in comparison with epithelioid mesotheliomas of the pleura. *Am J Surg Pathol* 2003; **27**: 150-158. (PMID: 12548160)
11. Rump A, Morikawa Y, Tanaka M, Minami S, Umesaki N, Takeuchi M, Miyajima A. Binding of ovarian cancer antigen CA125/MUC16 to mesothelin mediates cell adhesion. *J Biol Chem* 2004; **279**: 9190-9198. (PMID: 14676194)
12. Bera TK, Pastan I. Mesothelin is not required for normal mouse development or reproduction. *Mol Cell Biol* 2000; **20**: 2902-2906. (PMID: 10733593)
13. Hassan R, Bullock S, Premkumar A, Kreitman RJ, Kindler H, Willingham M, Pastan I. Phase I study of SS1P, a recombinant anti-mesothelin immunotoxin for targeted therapy of mesothelin expressing mesotheliomas, ovarian and pancreatic cancer [meeting abstract]. *J Clin Oncol* 2007; **25**: 3553.
14. Thomas AM, Santarsiero LM, Lutz ER, Armstrong TD, Chen YC, Huang LQ, Laheru DA, Goggins M, Hruban RH, Jaffee EM. Mesothelin-specific CD8(+) T cell responses provide evidence of in vivo cross-priming by antigen-presenting cells in vaccinated pancreatic cancer patients. *J Exp Med* 2004; **200**: 297-306. (PMID: 15289501)
15. Nicolaidis NC, Sass PM, Grasso L. Monoclonal antibodies: a morphing landscape for therapeutics. *Drug Dev Res* 2006; **67**: 781-789.
16. Chowdhury PS, Viner JL, Beers R, Pastan I. Isolation of a high-affinity stable single-chain Fv specific for mesothelin from DNA-immunized mice by phage display and construction of a recombinant immunotoxin with anti-tumor activity. *Proc Natl Acad Sci U S A* 1998; **95**: 669-674. (PMID: 9435250)
17. Chowdhury PS, Pastan I. Analysis of cloned Fvs from a phage display library indicates that DNA immunization can mimic antibody response generated by cell immunizations. *J Immunol Methods* 1999; **231**: 83-91. (PMID: 10648929)
18. Fransson J, Borrebaeck CA. The nuclear DNA repair protein Ku70/80 is a tumor-associated antigen displaying rapid receptor mediated endocytosis. *Int J Cancer* 2006; **119**: 2492-2496. (PMID: 16929494)
19. Gubbels JA, Belisle J, Onda M, Rancourt C, Migneault M, Ho M, Bera TK, Connor J, Sathyanarayana BK, Lee B, Pastan I, Patankar MS. Mesothelin-MUC16 binding is a high affinity, N-glycan dependent interaction that facilitates peritoneal metastasis of ovarian tumors. *Mol Cancer* 2006; **5**: 50. (PMID: 17067392)
20. Jain RK. Normalizing tumor vasculature with anti-angiogenic therapy: a new paradigm for combination therapy. *Nat Med* 2001; **7**: 987-989. (PMID: 11533692)
21. Holden SA, Lan Y, Pardo AM, Wesolowski JS, Gillies SD. Augmentation of antitumor activity of an antibody-interleukin 2 immunocytokine with chemotherapeutic agents. *Clin Cancer Res* 2001; **7**: 2862-2869. (PMID: 11555604)
22. Miers L, Lamborn K, Yuan A, Richman C, Natarajan A, DeNardo S, DeNardo G. Does paclitaxel (Taxol) given after (111)In-labeled monoclonal antibodies increase tumor-cumulated activity in epithelial cancers? *Clin Cancer Res* 2005; **11**: 7158s-7163s. (PMID: 16203816)
23. Jaffee EM, Schutte M, Gossett J, Morsberger LA, Adler AJ, Thomas M, Greten TF, Hruban RH, Yeo CJ, Griffin CA. Development and characterization of a cytokine-secreting pancreatic adenocarcinoma vaccine from primary tumors for use in clinical trials. *Cancer J Sci Am* 1998; **4**: 194-203. (PMID: 9612602)
24. Embuscado EE, Laheru D, Ricci F, Yun KJ, de Boom Witzel S, Seigel A, Flickinger K, Hidalgo M, Bova GS, Iacobuzio-Donahue CA. Immortalizing the complexity of cancer metastasis: genetic features of lethal metastatic pancreatic cancer obtained from rapid autopsy. *Cancer Biol Ther* 2005; **4**: 548-554. (PMID: 15846069)

Materials and methods

Cells

NCI-H226 and OVCAR-3 cells were purchased from the American Type Culture Collection (ATCC). IGROV-1 cells were obtained from the NCI cell collection. Panc 10.05 cells were previously described (23). Panc 3.27 and Panc 3.014 cells were generated in Dr. Elizabeth Jaffee's laboratory. A431-K5 cells were derived from A431, an epidermoid carcinoma cell line that has been stably transfected with a plasmid encoding mesothelin (6). A431-K5-TEM cells were derived in Dr. Grasso's laboratory from A431-K5 cells by transfection with an expression vector containing the full-length endosialin/TEM1 cDNA.

FACS analyses

Cells were harvested in cell dissociation solution (Invitrogen), washed, and resuspended in ice-cold PBS containing 1% FBS. Primary antibodies were added at 10 µg/mL and incubated for 1 hour on ice. Next, the cells were washed with PBS, incubated with FITC-conjugated goat-anti-human secondary antibody (Southern Biotech, Birmingham, AL) diluted to 1 µg/mL, washed as above and analyzed on an EasyCyte Flow Cytometer (Guava Technologies, Hayward, CA).

Immunohistochemistry

Samples of snap frozen pancreatic cancer were obtained from pancreaticoduodenectomy specimens or from tissues harvested in accordance with the Gastrointestinal Cancer Rapid Medical Donation Program at The Johns Hopkins Hospital (24). Five micron sections were cut from each tissue onto sterile glass slides and fixed in 100% ethanol for 1 minute, followed by rinsing in 1x TBS with Tween (TBST) for 5 minutes. Next, endogenous peroxidases were blocked by incubation with 3% hydrogen peroxide for 10 minutes followed by a second rinse in 1x TBST. Next, slides were incubated with a 0.5 µg/mL concentration of MORAb-009 antibody (Morphotek Inc., Exton, PA) diluted in 0.2 M Tris-HCL pH 7.5 for 1 hour at room temperature and again rinsed in 1x TBST for 5 minutes. Immunolabeling was detected using the LSAB+ Kit (DAKO) according to the manufacturer's instructions. Slides were counterstained in hematoxylin for 30 seconds and coverslipped. Staining patterns were evaluated by two of the authors (S.M. and C.I.D.) as negative (0), weakly positive (1+), positive (2+) and strongly positive (3+), and the percent labeling was evaluated on a 10-tiered scale ranging from 10% to 100%.

Internalization of MORAb-009

The human cell lines NCI-H226 and IGROV-1 were cultured in complete RPMI 1640 medium (1 mM sodium pyruvate, 5 U/mL penicillin, 5 µg/mL streptomycin, 2 mM L-glutamine) containing 10% FBS (Invitrogen) at 37°C, in a humidified atmosphere containing 5% CO₂. One day prior to addition of antibodies, cells were collected by trypsinization and plated at a density of 500 cells/well in a 96-well tissue culture plate (Nalgene Nunc) in complete RPMI 1640 medium. MORAb-009, polyclonal, saporin-conjugated goat anti-human antibodies (Hum-ZAP, Advanced Targeting Systems) or control antibodies were mixed in various ratios and incubated for 30 minutes at room temperature, prior to addition to cultured cells. Cells were incubated at 37°C, in a humidified atmosphere containing 5% CO₂, for 5 days prior to harvesting. Relative changes in cell numbers were quantified by the sulforhodamine B method. Briefly, cells were killed and fixed for one hour on ice by addition of ice-cold 100% trichloroacetic acid (TCA) directly into the growth medium, to a final concentration of 10%. TCA was removed by washing fixed cells 5 times using distilled, deionized water, and allowed to dry in open atmosphere. 100 µL of a solution of 0.4% sulforhodamine B in distilled, deionized water were added to each well, and incubated for 10 minutes. The wells were washed 4 times using 1% acetic acid and allowed to dry in open atmosphere. The resulting precipitate was dissolved in 100 µL of 10 mM Tris-Cl pH 8.0, and the absorbance at 540 nm was measured with a microplate reader. Relative cell numbers were determined by interpolation from a standard curve constructed by measuring absorbance of known cell numbers from each cell line, deposited in the bottom of a

96-well tissue culture plate. Results were expressed as % growth, by comparison to untreated cells.

ADCC assay

Donor peripheral blood mononuclear cells (PBMCs) used as effector cells were thawed and kept overnight in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal calf serum. The cells were resuspended in medium at a concentration of 10⁷ cells/mL. The tumor cells used as target cells were detached from the culture flask and 10⁶ cells in 100 µL fetal calf serum were labeled with 100 µCi (3.7 MBq) ⁵¹Cr for 2 hours at 37°C. Alternatively, lysing of cells was monitored by a standard lactate dehydrogenase (LDH) release assay following the manufacturer's instructions (Roche Applied Science, Indianapolis, IN). Cells were washed three times with 5 mL of medium and resuspended in medium at a concentration of 10⁵ cells/mL. Fifty microliters of the tumor cells were seeded in V-bottom 96-well culture plates. Cells were then incubated with 50 µL medium containing MORAb-009 or control antibody at concentrations indicated in the text. After 30 minutes of incubation at 37°C, 50 µL of the PBMCs were seeded in V-bottom 96-well culture plates at various target-effector cell ratios (1:0, 1:25, 1:50 and 1:100) and the plates were further incubated for 18 hours at 37°C. The release of ⁵¹Cr in the supernatant was determined in a gamma counter. Each measurement was carried out in triplicate.

CA125/MUC16-mesothelin interaction assay

OVCAR-3 cells (1 x 10⁵) were seeded in triplicate in microplates, incubated overnight at 37°C/5% CO₂ and allowed to form a monolayer. The following day, A431-K5-TEM cells (mesothelin+/TEM1+) were harvested and loaded with Calcein AM cell dye (Invitrogen Corp., Carlsbad, CA) as per the instruction manual. Labeled cells (2 x 10⁵) were pre-incubated for 1 hour at 4°C with MORAb-009 or control antibodies at a final concentration indicated in the figure legends. Both normal human polyclonal IgG (Jackson ImmunoResearch, WestGrove, PA) and anti-TEM1 antibody (Morphotek Inc., Exton, PA) were included to control for the specificity of the MORAb-009 inhibition of the mesothelin-MUC16 interaction. The OVCAR-3 monolayers were washed once with 200 µL 10% complete RPMI (cRPMI) and the cells/antibody mixtures added to triplicate wells for 45 minutes at 4°C. Wells were gently washed five times with 200 µL PBS by inverting the plate on paper towels. Cell adherence and its inhibition by MORAb-009 were quantitated using the MetaMorph Fluorescence Imaging System (ver.6, Molecular Dynamics, Downingtown, PA).

In vivo efficacy studies

Gemcitabine and Taxol® were provided by the Division of Veterinary Resources (NIH). A431-K5 cells were grown in DMEM with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/mL penicillin G, 100 µg/mL streptomycin and 750 µg/mL G418. Tumor experiments evaluating MORAb-009 in combination with gemcitabine were conducted using A431-K5 xenografts in nude mice. Four to six week old female athymic nude mice (National Cancer Institute-Frederick Animal Production Area, Frederick, MD) were housed in microisolator cages for the course of the experiment. The research protocol was approved and the mice were maintained as per institutional guidelines of the NIH. Two million A431-K5 cells were inoculated subcutaneously into the right flank of the mice. Tumor dimensions were determined using calipers and the tumor volume (mm³) was calculated by the formula: length

$\times (\text{width})^2 \times 0.4$. Treatment was initiated when tumors reached approximately 50 mm^3 in size. The different treatment regimens included: gemcitabine (80 mg/kg) alone via i.p. injection on days 7, 9 and 11 after tumor inoculation; MORAb-009 or Rituximab (50 mg/kg) alone given i.v. on days 7, 9 and 11 after tumor inoculation; gemcitabine (80 mg/kg) i.p. on days 7, 9 and 11 in combination with either MORAb-009 or Rituximab (50 mg/kg) i.v. on days 7, 9, 11, 13, 15 and 17 after tumor inoculation. Mice were sacrificed when the tumors reached approximately 500 mm^3 . To test the *in vivo* activity of MORAb-009 in combination with Taxol®, the same A431-K5 tumor xenograft model was used. On day 4 after tumor cell inoculation when the tumor size was approximately 60 mm^3 , mice ($n = 7$ per treatment group) were treated with either Taxol® 50 mg/kg i.p. on day 4, MORAb-009 50 mg/kg i.v. on days 4, 6 and 8 alone or the combination of Taxol® 50 mg/kg on day 4 and MORAb-009 50 mg/kg i.v. on days 4, 6, 8, 10, 12 and 14. Mice were sacrificed when the tumors reached approximately 500 mm^3 .

Non-human primate toxicity study

After preliminary dose-ranging studies, a 23-day intravenous infusion toxicity study in cynomolgus monkeys with a 28-day recovery period was conducted under Good Laboratory Practice (GLP). At the saline control and 15 mg/kg dose levels, three monkeys per sex were dosed with MORAb-009 twice per week over 23 days (seven total doses). At the 2 mg/kg dose level, two monkeys per sex were dosed at the same frequency. Two days after the last dose, two monkeys per sex were euthanized and their necropsy carried out. The remaining control and high dose monkeys in the respective recovery groups were observed for 28 additional days before euthanasia and necropsy. Clinical evaluations of the monkeys included the following: physical examinations pre- and post-dosing; daily clinical observations, including mortality checks; body weight measurements prior to study start, and twice weekly throughout the study; daily food consumption estimates beginning one week prior to treatment throughout the study; and hematology, coagulation, clinical chemistry and urinalysis measurements prior to study start and at study termination. Postmortem evaluations included the following: complete macroscopic examinations on all monkeys; selected organ weights; and complete histopathology examination. Blood was drawn before and after each dose, as well as during the recovery period for the determination of the toxicokinetics of MORAb-009 and for the detection of the possible generation of monkey anti-chimeric antibodies, which were determined by using ELISA.

Contact

Address correspondence to:

Luigi Grasso
210 Welsh Pool Road
Exton, PA 19341
USA
E-mail: grasso@morphotek.com