

# CAVELIER

ABOGADOS

www.cavelier.com  
cavelier@cavelier.com

EDIFICIO SISKI  
CARRERA 4 No. 72 - 35  
BOGOTÁ 8, COLOMBIA

249  
244  
Teléfono: (57-1) 347 3611  
Telefax: (57-1) 211 8660  
(57-1) 340 0860  
Fax in U.S.A.: (1-305) 381 9660

Señora Jefe de la  
**DIVISION DE NUEVAS CREACIONES**  
Superintendencia de Industria y Comercio  
E. S. D.

Trámite : 011 Evento : 379 Actuación : 44

Referencia : Expediente No. 04.120.665  
Asunto : Solicitud de Patente para **PRODUCTOS BIOLOGICOS**  
Solicitante : **CELLTECH R&D LIMITED**  
Fecha de Solicitud : 30 de Noviembre de 2004

1. Yo, Emilio FERRERO, obrando como apoderado del solicitante en el asunto antes identificado, me refiero al Oficio No. No. **12613** notificado por fijación en lista del 5 de Octubre de 2007.

2. Mediante mi memorial de fecha 28 de diciembre de 2007 solicité un plazo de 30 días para atender el Oficio No. **12613**.

3. Mediante el Oficio No. **12613** se puso en conocimiento del solicitante el informe que contiene las observaciones hechas por su Despacho sobre la presente solicitud de patente.

Atiendo a las observaciones de la manera siguiente:

### 3.1. CLARIDAD DE LA SOLICITUD Y UNIDAD DE INVENCION

Para subsanar las objeciones que sobre claridad y unidad de invención hizo su Despacho, presento nuevo pliego reivindicatorio con 8 reivindicaciones en el cual se han hecho los cambios sugeridos por su Despacho, que permiten apreciar claramente el objeto reivindicado en la presente solicitud de patente. Lo anterior, por cuanto las reivindicaciones se han limitado a los anticuerpos que tienen las regiones variables específicas del anticuerpo gL1gH7, el cual está debidamente sustentado en la descripción.

Adicionalmente, las reivindicaciones antiguas 2-7 y 9-10 han sido eliminadas de este pliego reivindicatorio.

De esta manera es claro que con las anteriores modificaciones al pliego reivindicatorio quedan subsanadas las objeciones que por claridad y unidad de invención hizo su Despacho, ya que ahora, dicho pliego trata un único concepto inventivo, a saber, anticuerpos que tienen las regiones variables del anticuerpo gL1gH7; permite determinar el alcance de la protección reivindicada y compararla frente al estado del arte, de acuerdo con las disposiciones de los artículos 25, 28 y 30 de la Decisión 486.

### 3.2. NIVEL INVENTIVO

Sobre este tema el señor Examinador sostiene que la invención solicitada por mi representada a la luz de D1 a D3 no goza de nivel inventivo, toda vez que el objeto de la solicitud podría resultar obvio para una persona del oficio normalmente versada en la materia y derivarse de manera evidente del estado de la técnica. Adicionalmente, el Despacho afirma que no se efectúan ensayos comparativos de la actividad de los anticuerpos derivados con respecto a los ya existentes.

A este respecto, es importante poner de presente que el examen de nivel inventivo está dirigido a determinar si la invención reivindicada había sido sugerida o enseñada en el estado del arte, de acuerdo con el conocimiento de un técnico medio en la materia. Para los anteriores efectos, el método problema-solución le indica al Examinador que al realizar el examen de nivel inventivo debe verificar las enseñanzas del arte previo sin adoptar la posición de un inventor que se preguntaría cómo solucionar el problema técnico en cuestión.

Lo anterior, por cuanto si se adopta la posición del inventor y se tiene conocimiento tanto del arte previo como de la forma como efectivamente se solucionó el problema técnico, la invención en todos los casos le resultará obvia y evidente.

Adicionalmente, se debe tener en cuenta que el mencionado método problema-solución también señala que los documentos del estado del arte frente a los cuales se debe efectuar el examen de nivel inventivo son aquellos que dentro del mismo campo técnico estén dirigidos a solucionar el mismo problema. Lo anterior, por cuanto estos son los documentos que se presume debieron ser de conocimiento del inventor para a partir de ellos formular su invención. Así los documentos relacionados con otro campo técnico o dirigidos a solucionar cualquier otro problema no serían relevantes para estos efectos en la medida que no harían parte del grupo de documentos de los cuales el inventor hubiera podido derivar de manera obvia y evidente su invención.

De acuerdo con lo anterior, procederemos a indicar las razones por las cuales los documentos citados no afectan el nivel inventivo de la presente solicitud de patente. Veamos:

A este respecto, respetuosamente ponemos de presente que los anticuerpos humanizados reivindicados en la presente solicitud muestran un efecto técnico sorprendente sobre los anticuerpos LL2 de D1 (Leung, S. et al, Molecular Immunology, 1995), que se hace evidente en su afinidad sorprendentemente mayor para CD22 que la versión humanizada del anticuerpo LL2 ("Epratuzumab"). Específicamente, los anticuerpos reivindicados tienen una afinidad de 235pM para el CD22 comparado con la afinidad para CD22 de 0.7nM para Epratuzumab. Así, la afinidad de los anticuerpos reivindicados es más de 3 veces mayor } Falso. que la afinidad del LL2 humanizado.

La afinidad del Epratuzumab para CD22 se ilustra en Camahan et al. (ver Anexo 4.4., LH col., "Resultado"). De la misma forma, la afinidad de los anticuerpos reivindicados para CD22 se ilustra por los resultados para el anticuerpo G5/44 presentado en DiJoseph, J. et al. (ver Anexo 4.5., página 1809 LH col bajo "Binding of CMC-544 to CD22"). El anticuerpo G5/44 de DiJoseph tiene las mismas secuencias de región variable que el anticuerpo gL1gH7. Esto es claro del documento posterior de DiJoseph (ver Anexo 4.6.), que

suministra la secuencia del anticuerpo G5/44 de la figura 5 y en la página 19, RH columna, segundo párrafo, última frase.

De manera sorprendente, las versiones injertadas humanizadas reivindicadas de los anticuerpos 5/44 tienen una afinidad aun mayor para el CD22 que el anticuerpo 5/44 quimérico del cual ellos se derivan (ver Figura 8 de la presente solicitud, que muestra la afinidad del 5/44 quimérico para CD22 es de 400 pM; también ver los resultados en la figura 12 y en la descripción de la presente solicitud, que muestran que el injerto gL1gH7 se desarrollo mas efectivamente que el quimérico 5/44 al competir contra el murino padre). Esto fue inesperado porque el proceso de humanización generalmente da como resultado la reducción de la afinidad comparada por el quimérico padre, mas no un incremento.

Adicionalmente, el anticuerpo LL2 descrito en D1 se une al epitopo B en CD22 (ver Anexo 4.6. página 22, RH col., 1er párrafo completo). En contraste, los anticuerpos de la presente invención se unen al epitopo A (ver Anexo, página 11, RH col.). Por lo tanto, D1 no suministra ninguna motivación para generar un anticuerpo que se una a éste epitopo.

Inf. posteri-  
or.

Mas aun, las secuencias del anticuerpo de las regiones variables el anticuerpo reivindicado no se puede considerar como derivables de manera obvia de D1, toda vez que como se mencionó anteriormente, D1 no describe un anticuerpo que se una al epitopo A sobre el CD22 humano con una afinidad de 235pM ni suministra ninguna guía de cómo producir tal anticuerpo.

Por su parte, ni D2 ni D3 enseñan las secuencias de los anticuerpos reivindicados y así estos documentos no remedian ninguna deficiencia de D1. Consecuentemente, la materia objeto reivindicada involucra nivel inventivo.

En vista de lo antes expuesto, no es posible objetar la presente invención con base en los documentos D1 a D3, por cuanto las enseñanzas, expresas o tácitas, del estado del arte,

tal y como lo exige el método problema-solución, ni anticipan la materia de la presente invención, es decir, los anticuerpos que tienen las regiones variables específicas del anticuerpo gL1gH7; ni dan sugerencia con respecto al efecto inesperado de tal materia, es decir, afinidad significativamente mejorada para el CD22 humano comparado con la técnica anterior. Por lo cual no se puede considerar que dichos documentos afecten de algún modo el nivel inventivo de la presente invención.

4. **ANEXOS:**

- 4.1. Nuevo pliego reivindicatorio con 8 reivindicaciones;
- 4.2. Formulario de modificaciones;
- 4.3. Recibo por valor de \$103.000 por concepto de modificaciones en solicitud pendiente.
- 4.4. Carnahan et Al "Epratuzumab, a Humanized Monoclonal Antibody Targeting CD22: Characterization of In Vitro Properties". Clinical Cancer Research, Vol 9, 2982s-3990s. September 1, 2003.
- 4.5. DiJoseph et Al "Antibody-targeted Chemotherapy with CMC-544: a CD22-targeted immunoconjugate of calicheamicin for the treatment of B-lymphoid malignancies". Blood, Vol. 103(5), 1807-1814, 2004.
- 4.6. DiJoseph et Al, "Antibody-targeted Chemotherapy of B-cell lymphoma using calicheamicin conjugated to murine or humanized antibody against CD22". Cancer Immunol Immunother, 54:11-24, 2005.

Señora Jefe,



**EMILIO FERRERO**  
T.P.A. No. 31.929



**Industria y Comercio**  
SUPERINTENDENCIA

Espacio reservado para el adhesivo de radicación

## FORMULARIO UNICO DE CORRECCIONES Y MODIFICACIONES

|                                                                                                                                                                                                                                                                                                                                                    |                                                                                                                                                                                                                                                                                                                                                           |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>1</b> SOLICITUD DE:                                                                                                                                                                                                                                                                                                                             |                                                                                                                                                                                                                                                                                                                                                           |
| <input type="checkbox"/> Corrección de Error Material <input checked="" type="checkbox"/> Modificación a una solicitud                                                                                                                                                                                                                             |                                                                                                                                                                                                                                                                                                                                                           |
| <b>2</b> SOLICITANTE                                                                                                                                                                                                                                                                                                                               | Nombre: <b>CELLTECH R&amp;D LIMITED</b><br>Sociedad constituida y existente bajo las leyes de Inglaterra.<br>Dirección: 208 Bath Road,<br>Slough,<br>Berkshire SL1 3WE,<br>Inglaterra<br>Domicilio: Slough,<br>Berkshire,<br>Inglaterra<br>Teléfono: 546 09 70 Fax: 249 40 70<br>E-mail: <a href="mailto:clientes@cavelier.com">clientes@cavelier.com</a> |
|                                                                                                                                                                                                                                                                                                                                                    | <b>IDENTIFICACION</b><br>C.C. <input type="checkbox"/> NIT <input type="checkbox"/><br>C.E. <input type="checkbox"/> Otro <input type="checkbox"/><br>Número: _____                                                                                                                                                                                       |
| <b>3</b> REPRESENTANTE O APODERADO                                                                                                                                                                                                                                                                                                                 | Nombre: <b>Emilio FERRERO</b><br>Dirección: Carrera 4ª No. 72-35<br>Teléfono: 347 36 11 Fax: 217 92 11<br>E-mail: <a href="mailto:cavelier@cavelier.com">cavelier@cavelier.com</a>                                                                                                                                                                        |
|                                                                                                                                                                                                                                                                                                                                                    | <b>IDENTIFICACION</b><br>C.C. <input checked="" type="checkbox"/> NIT <input type="checkbox"/><br>C.E. <input type="checkbox"/> Otro <input type="checkbox"/><br>Número: <u>438.019 TP 31.929</u>                                                                                                                                                         |
| <b>4</b> Número de expediente: <u>04.120.665</u>                                                                                                                                                                                                                                                                                                   |                                                                                                                                                                                                                                                                                                                                                           |
| <b>5</b> Descripción de la corrección o modificación solicitada:<br><u>Presento nuevo capítulo reivindicatorio compuesto por 8 reivindicaciones con el fin de superar las objeciones hechas por su Despacho a la presente solicitud de patente y pido que el examen de patentabilidad sea realizado sobre este nuevo capítulo reivindicatorio.</u> |                                                                                                                                                                                                                                                                                                                                                           |
| <b>6</b> Comprobante de pago No. <u>08-11,218</u> Fecha: <u>11 de febrero de 2008</u>                                                                                                                                                                                                                                                              |                                                                                                                                                                                                                                                                                                                                                           |
| <b>7</b> Anexos<br><input checked="" type="checkbox"/> Comprobante de pago de la tasa por \$103.000.00<br><input type="checkbox"/> Poderes, si fuere el caso<br><input type="checkbox"/> Documento que acredita la existencia y representación legal de las partes, cuando sean personas jurídicas.                                                |                                                                                                                                                                                                                                                                                                                                                           |
| <b>8</b> NOMBRE: <b>EMILIO FERRERO</b>                                                                                                                                                                                                                                                                                                             | FIRMA: <u></u>                                                                                                                                                                                                                                                                                                                                            |
| C.C. 438.019 de Usequén T.P.A. 31.929                                                                                                                                                                                                                                                                                                              |                                                                                                                                                                                                                                                                                                                                                           |

**REIVINDICACIONES**

- ① Una molécula de anticuerpo que tiene especificidad para el CD22 humano, que comprende la región variable de cadena ligera 5/44-gL1 (SEQ ID No: 19) y la región variable de cadena pesada 5/44-gH7 (SEQ ID No: 27).
2. Una molécula de anticuerpo de acuerdo a la reivindicación 1 que tiene una cadena ligera que comprende o que consiste de la secuencia dada en la SEQ ID No: 28 y la cadena pesa <sup>lida</sup> que comprende o que consiste de la secuencia dada en la SEQ ID No: 30.
3. Una secuencia de ADN que codifica una cadena pesada y/o la cadena ligera de la molécula de anticuerpo de acuerdo a la reivindicación 1 o reivindicación 2.
4. Un vector de clonación o de expresión que comprende una secuencia de ADN de acuerdo a la reivindicación 3.
5. Una célula huésped que comprende un vector de clonación o de expresión de acuerdo a la reivindicación 4.
6. Una composición terapéutica o diagnóstica que comprende la molécula de anticuerpo de la reivindicación 1 o la reivindicación 2 o una secuencia de ADN en la reivindicación 3. *No patentable*
7. Una composición terapéutica o diagnóstica de acuerdo a la reivindicación 6, que comprende un excipiente, diluyente o portador farmacéuticamente aceptable.
8. Una composición terapéutica o diagnóstica de acuerdo a la reivindicación 6 o reivindicación 7, que comprende adicionalmente una célula anti-T, anti-IFN $\gamma$  o anticuerpos anti-LPS, o xantinas.

## Epratuzumab, a Humanized Monoclonal Antibody Targeting CD22: Characterization of *in Vitro* Properties<sup>1</sup>

Josette Carnahan,<sup>2</sup> Paul Wang, Richard Kendall, Ching Chen, Sylvia Hu, Tom Boone, Todd Juan, Jane Talvenheimo, Silvia Montestruque, Jilin Sun, Gary Elliott, John Thomas, John Ferbas, Brent Kern, Robert Briddell, John P. Leonard, and Alessandra Cesano

Amgen, Inc., Thousand Oaks, California 91320 (J. C., P. W., R. K., C. C., S. H., T. B., T. J., J. T., S. M., J. S., G. E., J. T., J. F., B. K., R. B., A. C.), and Center for Lymphoma and Myeloma and Division of Hematology and Oncology, Weill Medical College of Cornell University and New York Presbyterian Hospital, New York, New York 10021 (J. P. L.)

### Abstract

**Purpose:** Epratuzumab is a novel humanized anti-human CD22 IgG1 antibody that has recently shown promising clinical activity, both as a single agent and in combination with rituximab, in patients with non-Hodgkin's lymphomas (NHL). In an attempt to better understand the mode of action of epratuzumab, the antibody was tested *in vitro* in a variety of cell-based assays similar to those used to evaluate the biological activity of other therapeutic monoclonal antibodies, including rituximab. In this report, we present epratuzumab activities as they relate to binding, signaling, and internalization of the receptor CD22.

**Methods:** Chinese hamster ovary-expressed CD22 extracellular domain was used to measure epratuzumab affinity on Biacore. CD22 receptor density and internalization rate were measured indirectly using a monovalently labeled, noncompeting (with epratuzumab) anti-CD22 antibody on Burkitt lymphoma cell lines, primary B cells derived from fresh tonsils, and B cells separated from peripheral blood samples obtained from patients with chronic lymphocytic leukemia or healthy volunteers. Epratuzumab-induced CD22 phosphorylation was measured by immunoprecipitation/Western blot and compared with that induced by anti-IgM stimulation.

**Results:** Epratuzumab binds to CD22-extracellular domain, with an affinity of  $K_D = 0.7$  nM. Binding of epratuzumab to B cell lines, or primary B cells from healthy individuals and patients with NHL, results in rapid internalization of the CD22/antibody complex. Internalization appears to be faster at early time points in cell lines than in primary B cells and NHL patient-derived B cells, but the

maximum internalization reached is comparable for all B cell populations after several hours of treatment and appears to reach saturation at antibody concentrations of 1–5  $\mu$ g/ml. Finally, epratuzumab binding results in modest but significant CD22 phosphorylation.

**Conclusions:** Epratuzumab represents an excellent anti-CD22 ligating agent, highly efficacious in inducing CD22 internalization, and can induce phosphorylation. Although we cannot unequivocally demonstrate here that epratuzumab-induced internalization and signaling of CD22 directly contribute to its therapeutic efficacy, these properties are the fundamental characteristics of the target CD22 and its interaction with epratuzumab. Similar results were observed when epratuzumab was tested *in vitro* on Burkitt B cell lines as well as on primary normal B cells and neoplastic B cells separated from fresh peripheral blood samples from patients with chronic lymphocytic leukemia.

### Introduction

CD22 is a 135-kDa transmembrane sialoglycoprotein and a member of the immunoglobulin superfamily. Its expression is restricted to lymphocytes of the B cell lineage and is highly developmentally regulated: CD22 is present in the cytoplasm of pro- and pre-B cells and becomes detectable on the cell surface only at mature stages of B cell differentiation. Cell surface expression is lost during terminal differentiation into plasma cell and after B cell activation (1–3). CD22 is also expressed by the vast majority of B cell NHLs<sup>3</sup> (4). The CD22 molecule has multiple ligands because it binds to  $\alpha$ 2–6-linked sialic acid residues present on glycoproteins expressed by activated T and B cells, monocytes, neutrophils, erythrocytes, and activated endothelial cells (5). Although its function is not yet well understood, CD22 appears to be involved in the regulation of B cell activation through BCR signaling, (demonstrating both positive and negative roles *in vitro*) as well as in cell adhesion (6). *In vivo*, the important biological functions of this receptor have been demonstrated by genetic disruption of CD22. CD22-deficient mice have a shorter life span, a reduced number of mature B cells in the bone marrow and in circulation, and a chronic exaggerated antibody response to antigen and develop elevated levels of autoantibodies, suggesting a key role for CD22 in B cell development, survival, and function (1, 7–9).

Because the expression of CD22 is lineage restricted and, in most cases, is not lost during neoplastic transformation, it represents an attractive target for anti-NHL immunotherapeutic antibodies. Preclinical work with anti-CD22-“blocking” monoclonal antibodies (*i.e.*, monoclonal antibodies that prevent CD22

<sup>1</sup> Presented at the “Ninth Conference on Cancer Therapy with Antibodies and Immunocytotoxins,” October 24–26, 2002, Princeton, NJ. Supported by Amgen, Inc., Thousand Oaks, CA.

<sup>2</sup> To whom requests for reprints should be addressed, at Amgen, Inc., One Amgen Center Drive, Thousand Oaks, CA 91320. Phone: (805) 447-4469 Fax: (805) 375-8368; E-mail: jcarnahan@amgen.com.

<sup>3</sup> The abbreviations used are: NHL, non-Hodgkin's lymphoma; BCR, B cell receptor; ECD, extracellular domain; PE, phycoerythrin; CLL, chronic lymphocytic leukemia.

binding to its natural ligands) suggests that modulation of receptor activity has a selective cytotoxic effect on receptor-positive tumor cells (10). Interestingly, the same ligand blocking anti-CD22 monoclonal antibody can trigger primary B cell proliferation, suggesting that the consequences of engaging CD22 may differ depending on the B cell stage of differentiation. In animal models, anti-CD22 monoclonal antibodies can mediate antitumor effects (11, 12). Unlike the B cell antigen CD20, CD22 internalizes rapidly upon antibody binding, making it an ideal target for delivery of radioisotopes or toxins to malignant cells via monoclonal antibody conjugates (11, 13-15).

LL2 is a mouse anti-CD22 monoclonal antibody (originally named EPB/2) generated against the Raji Burkitt's lymphoma cell line (16). LL2 is highly selective for B cell tumors but lacks reactivity with Hodgkin's disease, other solid tumors, or non-lymphoid tissues (17). A humanized version of the LL2 antibody, epratuzumab, was developed to minimize the potential for immunogenicity and enhancing effector interactions during its development as a diagnostic and immunotherapeutic (18). As a single agent, epratuzumab has shown preliminary evidence of antitumor activity in patients with recurrent NHL, producing responses, including durable complete responses (19). Objective responses were seen in 9 of 51 indolent NHL patients (17.6%; 3 complete responses, 6 partial responses) with a median duration of response of 47+ (range, 11-99+) weeks and median time to progression of 103+ (35-107+) weeks by Kaplan-Meier estimate. Of 52 aggressive NHL patients, 5 achieved objective responses (10%; 3 complete responses, 2 partial responses), with median duration of response 38+ (13 to 38+) weeks and median time to progression 35+ (23 to 35+) weeks. All responses occurred in the follicular NHL and diffuse large B cell histologies (19). Anti-CD22 agents are likely to have mechanism(s) of action distinct from those of other cytotoxic agents as well as from immunotherapies targeting other B cell antigens (e.g., the anti-CD20 monoclonal antibody rituximab and anti-CD52 antibody alemtuzumab). Thus, they are theoretically good candidates for combination with other drugs in the treatment of B cell malignancies. Epratuzumab is currently being evaluated in combination with rituximab, and early results from an ongoing study suggest that the combination of the two antibodies is well tolerated and may result in improved clinical activity versus the single agents alone (20).

This paper reports the results of *in vitro* studies to further characterize the mechanism of action of epratuzumab by exploring its interaction with CD22.

## Materials and Methods

**Cell Lines and Reagents.** The CD22-expressing human Burkitt's lymphoma cell lines, Daudi, Ramos, Namalwa, and Raji, were obtained from the American Type Culture Collection (Manassas, VA). The cell lines were grown in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated FBS (HyClone, Logan, UT), 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). Cells were cultured at 37°C in a humidified 5% CO<sub>2</sub> incubator. F(ab')<sub>2</sub> fragments of goat antihuman IgG(Fc) were purchased from Rockland Immunochemicals, Gilbertsville, PA. Other re-

agents included: F(ab')<sub>2</sub> fragment of goat antihuman IgM, Fc<sub>50</sub> (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), sodium orthovanadate (Sigma-Aldrich, St. Louis, MO), complete protease inhibitor mixture tablets (Roche, Mannheim, Germany), rabbit polyclonal anti-CD22 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), horseradish peroxidase-conjugated antiphosphotyrosine Ab (clone 4G10; Upstate Biotechnology, Inc., Lake Placid, NY), FITC- and PE-conjugated antihuman CD22 [clone SHCL-1 (Leu14)] antibody (BD Biosciences, San Diego, CA; PE:antibody ratio, 1:1), FITC-conjugated antihuman CD19 (BD Biosciences), and QuantiBRITE PE-conjugated beads (BD Biosciences).

**Cloning of Soluble Human CD22 ECD.** The extracellular domain of human  $\beta$  CD22 (amino acids 1-687) was amplified from a human spleen Quick Clone cDNA library (Clontech, Palo Alto, CA) using standard PCR procedure. The PCR primers contained the consensus Kozak sequences (CCACC) and the specific restriction enzyme sites for subsequent cloning linked to the 5' and 3' end of the CD22ECD sequences. The expression vector used, pDSR $\alpha$ , was modified from the pSR $\alpha$  vector (21), with a dihydrofolate reductase cassette inserted downstream to the expression cassette driven by the SV40 early promoter.

**Expression and Purification of CD22 ECD in Mammalian Cells.** Chinese hamster ovary cells (CHO-) defective in dihydrofolate reductase, originally obtained from L. Chasin (Columbia University, New York, NY) were grown in complete medium (high-glucose DMEM supplemented with 5% FBS, 1% nonessential amino acids, 1% hypoxanthine-thymidine, and 1% glutamine-penicillin-streptomycin).

CHO- cells were transfected with 10 µg/dish of linearized DNA [pDSR $\alpha$  CD22 ECD (containing amino acids 1-687 of human CD22)] using the calcium phosphate method (Invitrogen). CD22 ECD expression was analyzed by Western blot of serum-free conditioned medium harvested from confluent 24-well cultures. Gels were run under reducing conditions, and blots were probed with a rabbit polyclonal anti-CD22 antibody (Santa Cruz Biotechnology, Inc.). Clones showing the highest expression were expanded and seeded in roller bottles for the production of CD22 ECD.

CD22 ECD was purified from CHO cell-conditioned medium. The concentrated medium was buffer exchanged to 30 mM Tris-HCl, pH 8.5, and applied to a Q-Sepharose column equilibrated in the same buffer. The column was eluted with a linear gradient from 0 to 1 M NaCl in 30 mM Tris-HCl, pH 8.5. Fractions containing CD22 ECD were pooled and applied to a hydroxyapatite column equilibrated in 150 mM NaCl-30 mM Tris-HCl, pH 8.5. Bound protein was eluted with a linear gradient from 0 to 100 mM sodium phosphate, pH 7.0, in 150 mM NaCl. The fractions containing CD22 ECD were pooled and dialyzed into PBS. The final protein concentration was determined by UV absorbance at 280 nm, using an extinction coefficient of 144,270 M<sup>-1</sup> cm<sup>-1</sup>. The sample was determined to be  $\geq 98\%$  pure by SDS-PAGE.

**Biacore Measurement of Epratuzumab Binding Affinity.** Both kinetic and equilibrium analyses of epratuzumab were performed on a Biacore 3000 (Biacore, Inc., Piscataway, NJ) with PBS and 0.005% P20 surfactant (Biacore, Inc.) as running buffer.

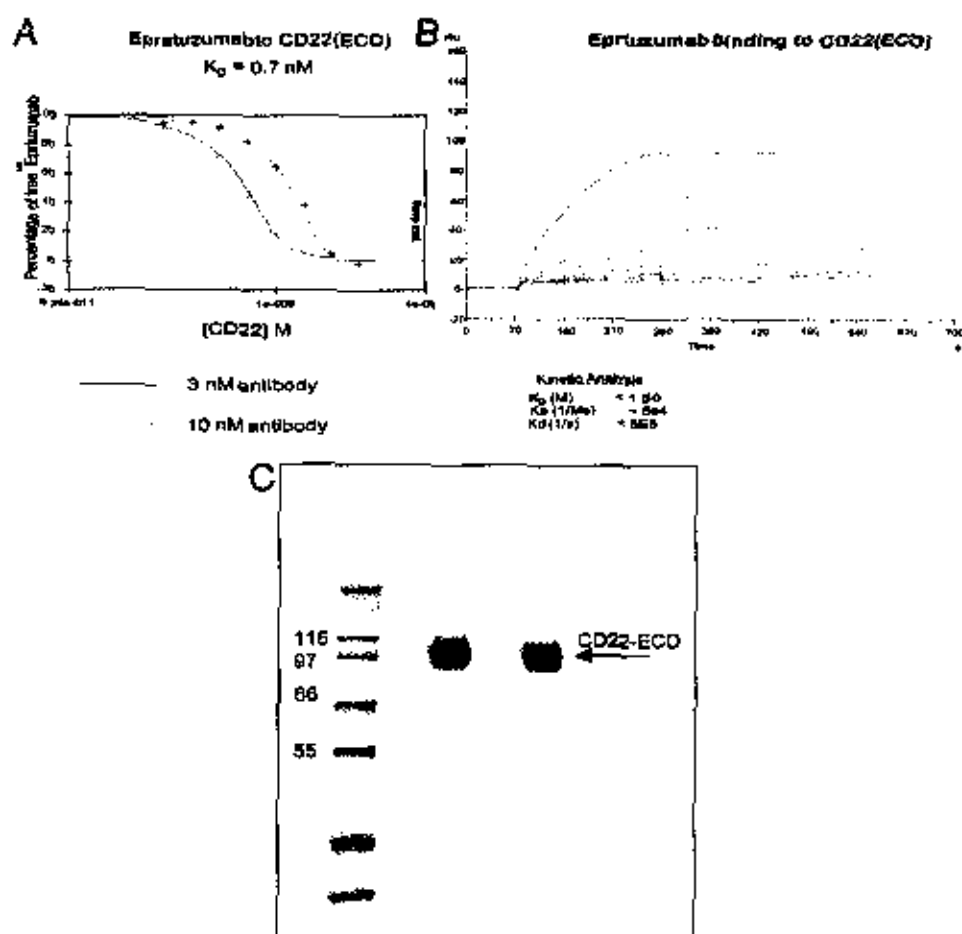


Fig. 1 Epratuzumab measured  $K_D$  on Biacore. Affinity of epratuzumab for CD22 ECD was determined by measuring binding of free epratuzumab to a Biacore column after competition with soluble CD22 ECD (A). B, kinetics of direct epratuzumab binding (0.8–200 nM) over a CD22 ECD surface, showing a very slow off rate. Cloned, Chinese hamster ovary-expressed, and purified CD22 ECD (as shown in panel C) on the blotted membrane with rabbit anti-CD22 was used for molecular characterization of epratuzumab binding.

To study the kinetics of epratuzumab/CD22 binding, CD22 ECD was immobilized to a research grade CM5 sensor chip (Biacore, Inc.) via primary amine groups using the Amine Coupling Kit (Biacore, Inc.) according to the manufacturer's suggested protocol. Resonance level was  $\sim 400$  Ru. The analysis was effected by injection of increasing concentrations (0.78–200 nM) of epratuzumab over the CD22 surface at a flow rate of 50  $\mu$ l/min for 3 min, and the dissociation reaction allowed for 15 min. Antibody binding kinetic parameters including  $k_a$  (association rate constant),  $k_d$  (dissociation rate constant), and  $K_D$  (dissociation equilibrium constant) were estimated using the BIA evaluation 3.1 computer program (Biacore, Inc.).

To analyze the equilibrium constants of epratuzumab-CD22 binding, CD22 ECD was immobilized to a CM5 chip (Resonance level = 2300 Ru) according to the manufacturer's suggested protocol. Epratuzumab at two different concentrations (3 nM and 10 nM) was incubated with increasing concentrations (0.04 nM to 80 nM) of CD22 in sample buffer (PBS + 0.005% P-20 + 0.1 mg/ml BSA) for  $> 2$  h to allow samples to reach equilibrium. Samples were then injected over the CD22 surface at 10  $\mu$ l/min for 30 min. In this system, the binding signal obtained is proportional to the free antibody in solution at equilibrium. The dissociation equilibrium constant ( $K_D$ ) was

obtained from nonlinear regression analysis of the competition curves using a dual-curve one-site homogeneous binding model (KinExA software; Sappidyne Instruments, Inc., Boise, ID).

#### CD22 Immunoprecipitation and Western Blot Analysis.

A total of  $10^7$  Daudi or Ramos cells (in 15 ml of RPMI 1640 plus 10% FBS) were treated with epratuzumab (5  $\mu$ g/ml); epratuzumab (5  $\mu$ g/ml) plus a cross-linking antibody (anti-human IgG(Fc) or F(ab')<sub>2</sub> fragments at 5  $\mu$ g/ml) or anti-human IgM F(ab')<sub>2</sub> fragments at 5  $\mu$ g/ml at 37°C for 15 min. Cells without antibody treatment were used as a negative control. After two washings with cold PBS, cells were centrifuged at  $200 \times g$  at 4°C for 8 min and resuspended in 400  $\mu$ l of lysis buffer (1% Triton X-100, 0.1% SDS in 1 $\times$  PBS with 2 mM sodium orthovanadate and protease inhibitor mixture). Cells were sonicated, kept on ice for 30 min, and centrifuged at  $16,000 \times g$  at 4°C for 10 min. Epratuzumab (3  $\mu$ g) and 20  $\mu$ l of protein G<sup>+</sup>/protein A-agarose beads (Oncogen Research Products, Boston, MA) were added to each supernatant and agitated overnight. Beads were then washed three times with lysis buffer. The immunoprecipitated proteins were size fractionated on SDS-PAGE and transferred to nitrocellulose. Membranes were analyzed by immunoblotting with horseradish peroxidase-conjugated antiphosphotyrosine antibody, treated with

stripping buffer (Pierce, Rockford, IL), and reprobed with a rabbit polyclonal anti-CD22.

**Primary B Cells from Fresh Tonsils and CLL Samples.** Tonsils were collected according to standard surgical procedures, including obtaining patient informed consent, and were shipped overnight in RPMI containing penicillin and streptomycin. Cells were gently mechanically dissociated in cold Hanks' balanced salt solution (Invitrogen), and mononuclear cells were purified using Ficoll-Hypaque (Amersham Biosciences) according to specification. Mononuclear cells were resuspended in Hanks' medium and washed with RPMI containing 10% FBS. Whenever the B cell:total cell ratio fell below 60%, further purification was performed using a T cell depletion kit from Dynal Biotech (Oslo, Norway).

Fresh CLL blood samples were collected in heparinized tubes from patients who had provided informed consent and were shipped overnight on ice. Ficoll-Hypaque purification of mononuclear cells was then performed as previously described. Cells were then immunostained with anti-CD19, anti-CD20, or anti-CD22 as described for FACS analysis and receptor quantification.

**Confocal Microscopy Study of Direct Epratuzumab Internalization.** FITC-labeled epratuzumab (2  $\mu$ g/ml) was used to label Daudi cells ( $10^6$  cells/ml) in PBS plus 0.5% BSA, on ice for 30 min. Cells were subsequently washed twice in PBS plus 0.5% BSA, plated in four-well chamber coverslips (Nalge Nunc International), and allowed to incubate for an additional 60 min at either 4°C or 37°C. Confocal images were recorded using an ACAS Ultima confocal microscope (Meridian Instruments, Inc., Okemos, MI) and represent 1- $\mu$ m sections through the center of a focal plane using a 100 $\times$  oil immersion objective.

**CD22, CD20, and CD19 Receptor Density Quantification.** Burkitt's lymphoma cell lines, enriched mononuclear cells from freshly dissociated tonsils, CLL, or normal healthy volunteer peripheral blood samples were used. One million cells per sample were blocked for nonspecific staining with 2% FBS + 1% human serum in PBS (0.05% sodium azide) for 20 min on ice. One microgram of anti-CD19-PE (clone SJ25C1), anti-CD20-PE (clone L27), or anti-CD22-PE (clone S-HCL-1 [Leu-14]) monoclonal antibody with the appropriate isotype controls was added for 30 min at 4°C in the dark. All antibodies were from BD Biosciences. Samples were washed with 2 ml of HBSS and centrifuged at 500  $\times$  g for 8 min at 10°C. The supernatants were discarded, and the cells were resuspended in 0.5 ml of FACS Lysing Solution (BD Biosciences) and immediately analyzed by flow cytometry (BD FACSCalibur). The CD19, CD20, and CD22 receptor numbers were converted from the standard fluorescence curve set by the QuantiBRITE PE fluorescence quantitation kit (BD Biosciences).

## Results

**Epratuzumab Binds to CD22 ECD on Biacore with a Measured Affinity of  $K_D = 0.7$  nM.** The binding activity of epratuzumab to CD22 ECD was measured on Biacore. In this system, in which CD22 ECD is immobilized, epratuzumab shows a relatively slow off rate ( $K_{off} < 10^{-3}$  1/s; Fig. 1B), leading to a  $K_D$  of 0.7 nM (Fig. 1A) as determined by equilibrium analysis. Although the on rate ( $k_{on}$ ) for epratuzumab is relatively

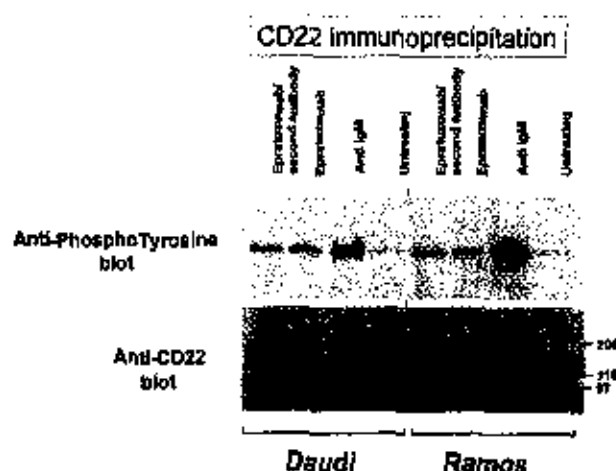


Fig 2. Epratuzumab induces phosphorylation of CD22. Daudi or Ramos cell lines were treated for 15 min at 37°C with a saturating dose of epratuzumab, epratuzumab and a cross-linker, or a B cell receptor activator, anti-IgM. Cells were lysed and CD22 was immunoprecipitated to examine the signaling potential of epratuzumab, using phosphotyrosine-specific antibody (top). To ensure equal CD22 loading, immunoblots were stripped and reprobed with a polyclonal rabbit anti-CD22 (bottom) (see "Materials and Methods").

slow, the resulting  $K_{13}$  is highly comparable with the reported 8 nM for rituximab (rituximab package insert).

**Epratuzumab Binding Results in CD22 Phosphorylation.** CD22 ligation has been reported by others to induce phosphorylation on its intracytoplasmic tail (10). Tyrosine phosphorylation of the CD22 cytoplasmic tail is also induced by BCR cross-linking. We therefore attempted (a) to detect whether epratuzumab could induce CD22 signaling and (b) to compare it with the CD22 tyrosine phosphorylation induced by B cell activation, using anti-IgM stimulation (Fig. 2).

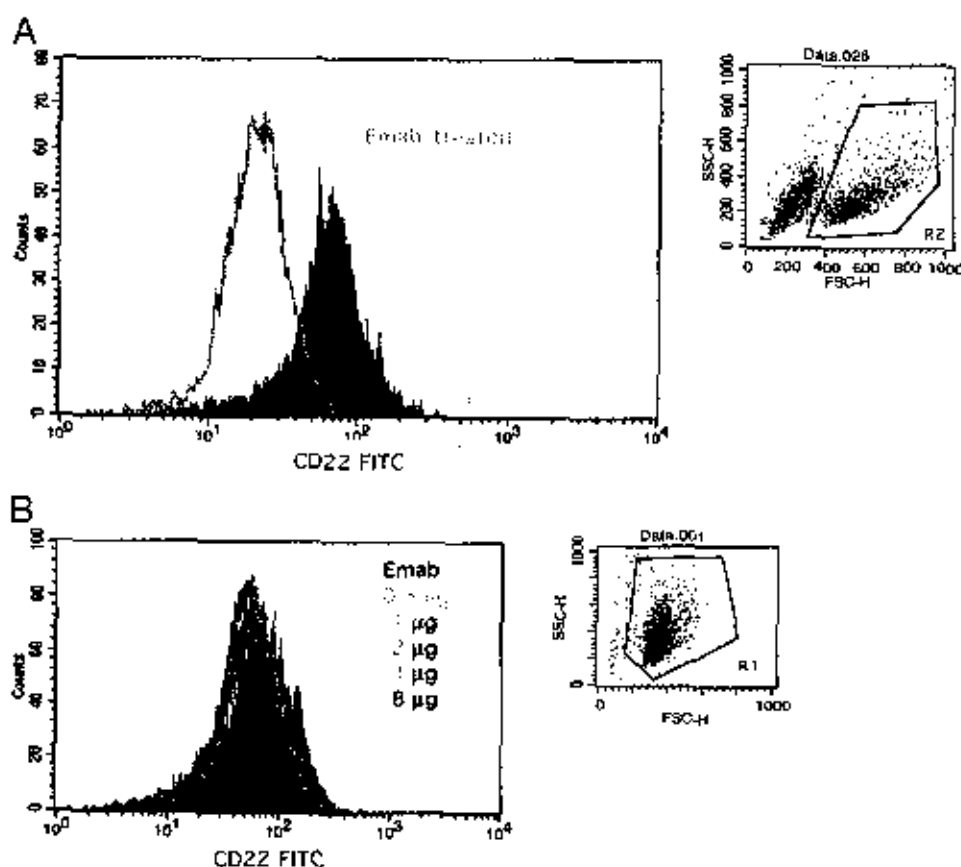
Daudi and Ramos Burkitt's lymphoma cell lines were treated for 15 min with saturating concentrations of epratuzumab. Immunoprecipitation-Western blot showed a significant increase in CD22 phosphorylation in both cell lines. Cross-linking epratuzumab using a secondary antibody did not result in higher level of phosphorylation. As expected, stimulation of the BCR using an activating anti-IgM antibody resulted in the highest CD22 phosphorylation level. Epratuzumab binding to tonsil-derived primary B cells also resulted in CD22 phosphorylation above resting level. However, the CD22 phosphorylation level in unstimulated tonsil cells was higher than in the unstimulated cell lines, decreasing the signal:noise ratio (data not shown).

**Epratuzumab Internalization Visualized by Confocal Microscopy.** In an effort to visually document the rapid, direct internalization of epratuzumab after binding to CD22, epratuzumab was directly labeled with FITC, and its binding to Daudi Burkitt lymphoma cells was monitored at 37°C during 60 min. As a control, Daudi cells were exposed to the same epratuzumab concentration but kept on ice to prevent internalization (Fig. 3, left). FITC-epratuzumab/CD22 internalization is evident from capping phenomena and punctate staining of endocytic vesicles (Fig. 3, arrows) visible in cells incubated at 37°C only.



Fig. 3 FITC-epratuzumab direct internalization. FITC-labeled epratuzumab was used to label Daudi tumor cells on ice for 30 min. Cells were subsequently washed and allowed to incubate for an additional 60 min at either 4°C (left) or 37°C (right). CD22 internalization is evident from capping and punctate staining of endocytic vesicles (arrows) visible in cells incubated at 37°C.

Fig. 4 CD22 internalization as measured by flow cytometry using a noncompeting anti-CD22 antibody. Daudi cells were incubated in the presence of 5  $\mu$ g/ml epratuzumab (Emab) for 20 h at 37°C and analyzed by flow cytometry for CD22 internalization (A) as observed by an apparent lowering of cell surface CD22, measured with the FITC-S-HCL1 anti-CD22 monoclonal antibody. B, evidence that adding increasing epratuzumab concentrations together with FITC-labeled S-HCL1 did not result in lowering the signal observed with FITC-S-HCL1 alone (solid blue trace). This confirms that these two anti-CD22 monoclonal antibodies recognize distinct and noncompeting epitopes on CD22 (FACS using the Daudi cell line). FSC-H, forward scatter; SSC-H, side scatter.



**Internalization of Epratuzumab Results in Quantifiable CD22 Cell Surface Decrease.** One particularly intriguing property of CD22 is its capacity to rapidly translocate from the cytoplasm to the cell surface on B cell stimulation on cell lines, (22) and to rapidly internalize upon antibody ligation (23). The result of such internalization is an apparent reduction in the number of cell surface CD22 binding sites (Fig. 4A), measurable using a noncompeting anti-CD22 (anti-CD22-PE [clone S-HCL-1 (Leu-14)] monoclonal antibody (24).

We confirmed that the anti-CD22 [clone S-HCL-1 (Leu-14)] monoclonal antibody did not compete with epratuzumab for CD22 binding and therefore could be used as a tool to independently measure cell surface CD22. As shown in Fig. 4B, increasing concentration of epratuzumab added together with S-HCL1-FITC labeled antibody did not result in a displacement of the signal observed with S-HCL1-FITC antibody alone.

To quantify cell surface CD22, the S-HCL1 antibody monovalently labeled with PE (conjugated by BD Biosciences)

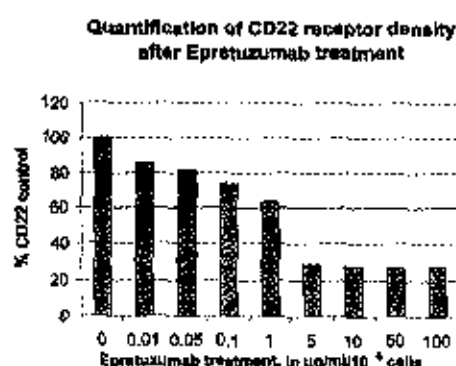


**Fig. 5** Quantification of CD22 receptor density after epratuzumab treatment. Daudi lymphoma cells ( $10^6$  cells/ml) were incubated with increasing concentrations of epratuzumab in RPMI supplemented with 10% FBS for 1 h at 37°C. Cells were then washed with cold PBS and immediately analyzed by FACS for CD22 receptor quantification as described. The resulting CD22 density as measured with QuantiBRITE beads and H-SCL1 PE labeled is shown as a percentage of total CD22 on untreated cells (100%). At 5 µg/ml, cells have reached the maximum level of internalization, which is 70% in this experiment.

was often used after cells were incubated with epratuzumab. This allowed indirect measurement of the CD22 receptor internalization rate and extent after a variety of treatments, without direct modification of epratuzumab.

Incubation of Daudi cells for 1 hour with epratuzumab resulted in a dose dependent increase in CD22 internalization (Fig. 5). The point of saturation was reached between 1 to 5 µg/ml/ $10^6$  cells (31.5 nM). At this time point, 30% of the original CD22 density (or 70% internalization) was observed. Similar results were obtained with other cell lines, such as Ramos, Raji, and Namalwa (data not shown), suggesting that the lack of total internalization may reflect more the endogenous turnover of the receptor (25) rather than expression of another CD22 isoform (i.e., the  $\alpha$  isoform of CD22, lacking domains 3 and 4 and therefore presumably not bound by epratuzumab).

Consistent with a dynamic equilibrium between rapid epratuzumab-induced CD22 internalization and trafficking to the cell surface of new receptor, prolonged incubation time with epratuzumab resulted in higher degree of internalization, reaching close to 80% after an overnight incubation, in both cell lines (Daudi) and primary tonsil-derived B cells (Fig. 6). At early time points, the Daudi cell line internalizes CD22 very rapidly, consistent with results reported previously for the Raji cell line (23). Internalization of the epratuzumab/CD22 complex on tonsil-derived primary B cells appears much slower. However, the extent and trend for increasing internalization over time did not differ significantly between the two cell populations after a 30-min incubation. To determine whether these observations could be extended to "fresh" lymphoma cells, mononuclear cells isolated by Ficoll-Hypaque from peripheral blood samples from patients with CLL were incubated with saturating concentrations of epratuzumab for 2 h, and CD22 expression was quantified at the end of the incubation time using the QuantiBRITE system (Table 1). Among the CLL and NHL samples tested, CD22 receptor density tends to be lower than in established lymphoma cell lines. However, even in these samples, evidence

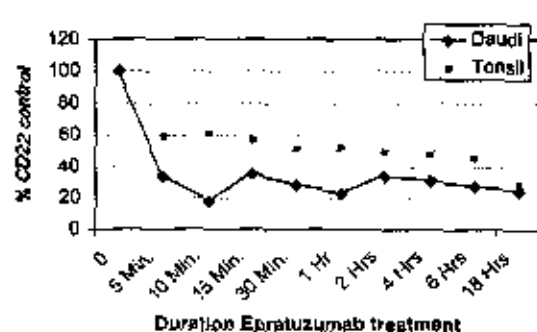


**Fig. 6** Epratuzumab-induced CD22 internalization kinetics. Daudi cells and tonsil-derived mononuclear cells were incubated with increasing incubation time in the presence of 25 µg/ml epratuzumab. At the end of each time point, cells were immunostained with anti-CD22 H-SCL1-PE only (Daudi) or anti-CD19-FITC and anti-CD22-PE (tonsil) for receptor quantification, as described. At early time points, the Daudi cell line is more efficacious in receptor internalization. After prolonged treatment, however (> 18 h), no internalization efficiency difference between cell lines and primary cells could be observed.

of CD22 internalization induced by epratuzumab was obtained after a 2-h incubation. For comparison, we tested in the same experiment the B lymphoma cell line Namalwa that expresses low levels of cell surface CD22. Namalwa cells, like Daudi cells, show a very fast and almost maximum level of internalization within minutes (data not shown), indicating that epratuzumab-mediated receptor internalization can occur very efficiently even with low receptor density, but the kinetics may differ significantly between cell lines and primary samples.

#### CD22 Reexpression after Epratuzumab Treatment

Because in the clinical setting patients will be exposed to epratuzumab for days ( $t_{1/2} = 23$  days), the effect of longer epratuzumab exposure was also explored on Daudi and Ramos cell lines by growing them in the presence of 10 µg/ml epratuzumab for up to 13 days. At each passage/feeding time, cells were counted, and viability was evaluated with trypan blue. No significant difference in cell density and viability could be detected between epratuzumab-treated or control cells (data not shown), suggesting that epratuzumab does not have a direct cytotoxic or cytostatic effect *in vitro*. At day 4 or 7 during the 13-day period, some cells were washed and transferred to non-epratuzumab-containing medium and cultured for an additional 9 or 6 days, respectively. On the last day, all groups of cells were stained with FITC-H-SCL1 anti-CD22 monoclonal antibody and analyzed by flow cytometry (Fig. 7). As shown in Fig. 7, prolonged exposure of cells to epratuzumab for up to 13 days did not result in total abrogation of CD22 cell surface staining. Surprisingly, both Daudi (Fig. 7, top) and Ramos (Fig. 7, bottom) cell lines did not show full recovery of CD22 expression even after a prolonged (6 and 9 days) period of recovery from epratuzumab treatment when compared with cells grown in control conditions without epratuzumab.

#### Discussion

CD22 is an appealing target for the development of novel treatments for B cell malignancies because of its expression, which is B cell restricted and developmentally regulated, and its

Table 1 CD22, CD20, and CD19 receptor density, and epratuzumab-induced CD22 internalization on NHL and tonsil primary B cells-derived compared to two Burkitt lymphoma cell lines

| Patient | Samples               | Receptor density |                 |        | % internalization |
|---------|-----------------------|------------------|-----------------|--------|-------------------|
|         |                       | CD22             | CD19            | CD20   |                   |
| 1       | CLL                   | 1,037            | ND <sup>b</sup> | ND     | 50                |
| 2       | CLL                   | 2,077            | 4,770           | 4,230  | 40                |
| 3       | NHL                   | 1,297            | 3,859           | 6,758  | ND                |
| 4       | Mantle cell lymphomas | 16,907           | ND              | ND     | 65                |
| 5       | CLL                   | 6,107            | ND              | ND     | 48                |
| 6       | Normal tonsils        | 11,326           | 3,482           | 12,485 | 40–50             |
| 7       | Normal tonsils        | 5,570            | 3,438           | 40,930 | 40–50             |
| 8       | Normal tonsils        | 6,574            | 8,201           | 13,584 | 40–50             |
| 9       | Healthy donor         | 20,047           | ND              | 24,312 | ND                |
|         | Daudi                 | 22,548           | 21,787          | 23,604 | 60                |
|         | Ramos                 | 5,187            | 6,309           | 3,584  | 50                |

<sup>a</sup> Primary B cells were enriched from freshly dissociated tonsils (sample 6–8) or blood collected from patients with B-cell malignancies (sample 1–5) or healthy volunteer (sample 9). Cells were immediately analyzed for receptor density quantification as described, or treated with saturating dose of epratuzumab, for 2 hours at 37°C.

<sup>b</sup> ND, not done.

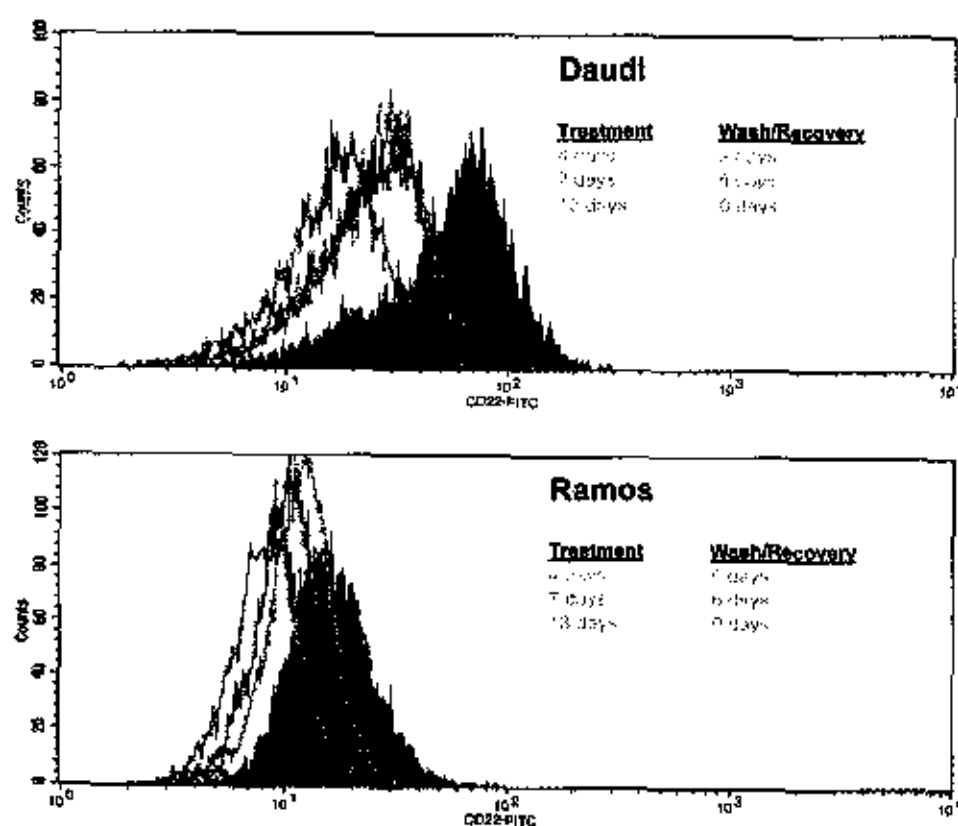


Fig. 7 Partial recovery of surface CD22 after epratuzumab treatment. Daudi or Ramos cell lines were grown in RPMI-10% FBS with added 20 µg/ml epratuzumab (or no added antibody for negative control) for up to 13 days. After 4 or 7 days treatment, cells were washed and allowed to recover in non-antibody-containing growth medium for 6–9 days. In all, the experiment lasted for 13 days, during which cell density was kept at  $10^6$  cells/ml, and viability was >95%. At the end of 13 days, all groups were analyzed by flow cytometry for CD22 expression using H-SCL1-FITC labeled (BD Biosciences), and the profiles were overlaid with that of untreated control cells (solid blue trace).

physiological functions as an adhesion molecule and a regulator of B cell receptor activation. One of the main functions of CD22 is to regulate B cell responses through recruitment of key signaling molecules to the antigen/receptor complex. Part of the BCR activation pathway includes tyrosine phosphorylation of the CD22 intracellular tail. However, CD22 ligand-mediated

signaling can also be achieved by engaging the CD22 ligand binding site (mapped to CD22, domains 1 and 2) (5).

Epratuzumab is a humanized monoclonal antibody directed against the CD22 molecule; the parental murine antibody (LL2) was originally developed by Goldenberg *et al.* by "classic" immunization of mice with Raji cells as a source of B cell

antigens. This report confirms unequivocally that epratuzumab binds to CD22 ECD. The epratuzumab  $K_D$  value measured in this study on Biacore is consistent with previously published data on epratuzumab binding affinity to Raji cells using radiolabeled LL2 competed out with cold LL2 (estimated  $K_D$  from these experiments would be in the low nM range) (26). This compares favorably with other monoclonal antibodies used in the treatment of B cell lymphomas; for instance, rituximab affinity is reported to be 8 nM (rituximab package insert).

Epratuzumab binding to CD22 does not block the ligand-binding site on CD22 (17). In fact, as opposed to the ligand blocking anti-CD22 monoclonal antibodies (19), epratuzumab did not show evidence of inducing apoptosis or growth arrest on lymphoma cell growth *in vitro* (data not shown). Because CD22 interacts with a variety of ligands (including CD45) and cellular partners (including T cells) (5, 27), the effect of modulating this receptor should be significant *in vivo* but might be difficult to demonstrate in an isolated context *in vitro*. For example, others have shown that blocking CD22 translocation by using beads coated with anti-CD22 antibodies can alter the B cell threshold of activation through BCR (28).

In addition to its signaling regulation of B cell activation, CD22 was shown by several groups to internalize rapidly on antibody ligation (29). In the majority of cases internalization was demonstrated using radiolabeled antibody. In this report, we confirm the results previously published by using FITC-labeled epratuzumab as well as by measuring "naked" epratuzumab binding/internalization indirectly. In particular, in our system using confocal microscopy the internalization of the epratuzumab/CD22 complex could be visualized as intracytoplasmic vesicles presumably of endosome-lysosome origin. The internalization was dose dependent and long lasting, without reaching reversibility at clinically meaningful concentrations. Moreover, epratuzumab prolonged incubation results, at a given dose, in a progressive decrease in CD22 surface level, presumably shifting the equilibrium between internalization and receptor turnover. However, total disappearance of CD22 from the cell surface was never observed. These results could be explained by the known coexpression of a shorter spliced variant of CD22, anti-CD22. This spliced mRNA minor variant found on human B cells is missing exons 5–8, resulting in CD22 ECD that lack domains 3 and 4 (30). This isoform therefore would not bear the epitope to which epratuzumab binds. However, using cell lines known to express various ratios of the two isoforms, similar apparent incomplete depletion of cell surface CD22 (data not shown) after prolonged epratuzumab incubation was observed, thus making the above hypothesis unlikely. Because in the clinical setting patients will be exposed to epratuzumab for a long period of time, we are now measuring the effect of epratuzumab treatment on CD22 mRNA expression as well as on time for complete recovery of cell surface CD22.<sup>4</sup> Reexpression of CD22 after prolonged epratuzumab treatment appears to follow slow kinetics; this was determined on two B cell lines grown for a different number of days in antibody-free medium after being exposed to epratuzumab for various periods of time. To our

surprise, even after cells were washed and grown for 4–9 more days in what should be essentially epratuzumab-free medium, the CD22 level did not fully recover compared with untreated cells. It is possible that the actual epratuzumab concentration in fresh medium is modified by the epratuzumab pool bound to cytoplasmic CD22 being released during cell division or that CD22 expression at the RNA level is down-regulated by the antibody treatment. This is an important point to address in future experiments, because it may have implications in epratuzumab dosing/scheduling in the clinic.

In conclusion, the CD22 molecule serves at least two important roles, *i.e.*, regulation of BCR activation (mostly negative) and involvement in mature B cell homing. Both are key functions that epratuzumab can modulate through induction of CD22 phosphorylation and CD22 internalization, respectively. Although difficult to demonstrate *in vitro*, these effects could have very different implications depending on the stage of maturation of B cells, and their compartment localization. For example, recent reports suggest adhesion molecules to be implicated in homing, dissemination, and survival of NHL cells (31, 32).

Overall the results presented here, although not directly answering the question of the *in vivo* mechanism of action of epratuzumab, do help to clearly differentiate this antibody from other monoclonal antibodies currently used in the treatment of NHL. This differentiation, at least theoretically, forms the basis for the clinical evaluation of combination therapies.

#### Acknowledgments

We thank Jamie Matthews for providing peripheral blood patient samples and Cooperative Tissue Network, Western Division, as a source of normal fresh tonsils.

#### References

1. Nitschke, L., Carsetti, R., Ocker, B., Kohler, G., and Lamers, M. C. CD22 is a negative regulator of B-cell receptor signalling. *Curr. Biol.*, 7: 133–143, 1997.
2. Saito, S., Tuscano, J. M., Inaoki, M., and Tedder, T. F. CD22 negatively and positively regulates signal transduction through the B lymphocyte antigen receptor. *Semin. Immunol.*, 10: 287–297, 1998.
3. Dörken, B., Moldenhauer, G., Pezzutto, A., Schwartz, R., Feljé, A., Kiesel, S., and Nadler, L. M. *HD39 (B3)*, a B lineage-restricted antigen whose cell surface expression is limited to resting and activated human B lymphocytes. *J. Immunol.*, 136: 4470–4479, 1986.
4. Knowles, D. M., Chadburn, A., and Iqbal, G. Immunophenotypic markers useful in the diagnosis and classification of hematopoietic neoplasms. In: D. M. Knowles (ed.), *Neoplastic Hematopathology*, pp. 73–95. Baltimore: Williams & Wilkins, 1992.
5. Engel, P., Nojima, Y., Rothstein, D., Zhou, L. J., Wilson, G. L., Kehrl, J. H., and Tedder, T. F. The same epitope on CD22 of B lymphocytes mediates the adhesion of erythrocytes, T and B lymphocytes, neutrophils, and monocytes. *J. Immunol.*, 150: 4719–4732, 1993.
6. Cyster, J. G., and Goodnow, C. C. Tuning antigen receptor signaling by CD22: integrating cues from antigens and the microenvironment. *Immunity*, 6: 509–517, 1997.
7. Orpoby, K. L., Andersson, K. B., Draves, K. E., Klaus, S. J., Farr, A. G., Kerner, J. D., Perlmutter, R. M., Law, C.-L., and Clark, E. A. CD22 regulates thymus-independent responses and the lifespan of B cells. *Nature*, 384: 634–637, 1996.
8. O'Keefe, T. L., Williams, G. T., Draves, S. L., and Neuberger, M. S. Hyperresponsive B cells in CD22-deficient mice. *Science (Wash. DC)*, 274: 798–801, 1996.

<sup>4</sup> Manuscript in preparation.

9. Sato, S., Miller, A. S., Inagaki, M., Bock, C. B., Jansen, P. J., Tang, M. L., and Tedder, T. F. CD22 is both a positive and negative regulator of B lymphocyte antigen receptor signal transduction: altered signaling in CD22-deficient mice. *Immunity*, 5: 551-562, 1996.
10. Tuscano, J. M., Agostino, R., Toscano, S. N., Tedder, T. F., and Kehrl, J. H. CD22 cross-linking generates B-cell antigen receptor-independent signals that activate the JNK/SAPK signaling cascade. *Blood*, 94: 1382-1392, 1999.
11. Kreitman, R. J., Hansen, H. J., Jones, A. L., FitzGerald, D. J., Goldenberg, D. M., and Pastan, I. *Pseudomonas* exotoxin-based immunotoxins containing the antibody LL2 or LL2-Fab' induce regression of subcutaneous human B-cell lymphoma in mice. *Cancer Res*, 53: 819-825, 1993.
12. Tuscano, J. M., DeNardo, G., Wims, T., Tedder, T. F., and Kehrl, J. H. Manipulation of CD22 signal transduction for the treatment of lymphoma. *Blood*, 94 (10 Suppl. 1, Part 1): 93a, 1999.
13. Ghetie, M.-A., Richardson, J., Tucker, T., Jones, D., Uhr, J. W., and Ytetta, E. S. Antitumor activity of Fab' and IgG-anti-CD22 immunotoxins in disseminated human B lymphoma grown in mice with severe combined immunodeficiency disease: effect on tumor cells in extranodal sites. *Cancer Res*, 51: 5876-5880, 1991.
14. Newton, D. L., Hansen, H. J., Mikulski, S. M., Goldenberg, D. M., and Rybak, S. M. Potent and specific antitumor effects of an anti-CD22-targeted cytotoxic ribonuclease: potential for the treatment of non-Hodgkin lymphoma. *Blood*, 97: 528-535, 2001.
15. Behr, T. M., Holler, E., Gratz, S., Wormann, B., Sharkey, R. M., Dunn, R. M., Hiddemann, W., Kolb, H.-J., Goldenberg, D. M., and Becker, W. CD22 is a suitable target molecule for detection and high-dose, myeloablative radioimmunotherapy with the monoclonal antibody LL2 in acute lymphatic leukaemia and Waldenström's macroglobulinemia. *Tumor Targeting*, 3: 32-40, 1998.
16. Pawlak-Byczkowska, E. J., Hansen, H. J., Dion, A. S., and Goldenberg, D. M. Two new monoclonal antibodies, EPB-1 and EPB-2, reactive with human lymphoma. *Cancer Res*, 49: 4568-4577, 1989.
17. Stein, R., Belisle, E., Hansen, H. J., and Goldenberg, D. M. Epitope specificity of the anti-B-cell lymphoma monoclonal antibody, LL2. *Cancer Immunol. Immunother.*, 37: 293-298, 1993.
18. Leung, S. O., Goldenberg, D. M., Dion, A. S., Pellegrini, M. C., Shevitz, J., Shih, L. B., and Hansen, H. J. Construction and characterization of a humanized, internalizing, B-cell (CD22)-specific, leukemia/lymphoma antibody, LL2. *Mol. Immunol.*, 32: 1413-1427, 1995.
19. Leonard, J. P., Coleman, M., Matthews, J. C., et al. Phase III trial of epratuzumab (humanized anti-CD22 antibody) in non-Hodgkin's lymphoma (NHL). *Blood*, 100: 358a, 2002.
20. Leonard, J. P., Coleman, M., Matthews, J. C., et al. Epratuzumab (anti-CD22) and rituximab (anti-CD20) combination immunotherapy for non-Hodgkin's lymphoma (NHL): preliminary response data. *Proc. Am. Soc. Clin. Oncol.*, 21: 266a, 2002.
21. Takebe, Y., Seiki, M., Fujisawa, J., Hoy, P., Yokota, K., Arai, K., Yoshida, M., and Arai, N. SR  $\alpha$  promoter: an efficient and versatile mammalian cDNA expression system composed of the simian virus 40 early promoter and the R-U5 segment of human T-cell leukemia virus type 1 long terminal repeat. *Mol. Cell. Biol.*, 8: 466-472, 1988.
22. Sherbina, N. V., Linsley, P. S., Myrdal, S., Grosmaire, L. S., Ledbetter, J. A., and Schieven, G. L. Intracellular CD22 rapidly moves to the cell surface in a tyrosine kinase-dependent manner following antigen receptor stimulation. *J. Immunol.*, 157: 4390-4398, 1996.
23. Shih, L. B., Lu, H. H.-Z., Xiang, H., and Goldenberg, D. M. Internalization and intracellular processing of an anti-B-cell lymphoma monoclonal antibody, LL2. *Int. J. Cancer*, 56: 538-545, 1994.
24. Schwartz, R., Stein, H., and Wang, C. Y. The monoclonal antibodies  $\alpha$  S-HCL 1 ( $\alpha$  Leu-14) and  $\alpha$  S-HCL 3 ( $\alpha$  Leu-M5) allow the diagnosis of hairy cell leukemia. *Blood*, 65: 974-983, 1990.
25. Shan, D., and Press, O. W. Constitutive endocytosis and degradation of CD22 by human B cells. *J. Immunol.*, 154: 4466-4475, 1995.
26. Leung, S. O., Shevitz, J., Pellegrini, A. S., Dion, A. S., Shih, L. B., Goldenberg, D. M., and Hansen, H. J. Chimerization of LL2, a rapidly internalizing antibody specific for B cell lymphoma. *Hybridoma*, 13: 469-476, 1994.
27. Stamenkovic, I., Sguri, D., Aruffo, A., Si, M. S., and Anderson, T. The B lymphocyte adhesion molecule CD22 interacts with leukocyte common antigen CD45RO on T cells and  $\alpha$  2-6 sialyltransferase, CD75, on B cells. *Cell*, 66: 1133-1144, 1991.
28. Doody, G. M., Justement, L. B., Delibrias, C. C., Matthews, R. J., Lin, J., Thomas, M. L., and Fearon, D. T. A role in B cell activation for CD22 and the protein tyrosine phosphatase SHP. *Science (Wash. DC)*, 269: 242-244, 1995.
29. Press, O. W., Farr, A. G., Borroz, K. I., Anderson, S. K., and Martin, P. J. Endocytosis and degradation of monoclonal antibodies targeting human B-cell malignancies. *Cancer Res*, 49: 4906-4912, 1989.
30. Wilson, G. L., Fox, C. H., Fauzi, A. S., and Kehrl, J. H. cDNA cloning of the B cell membrane protein CD22: a mediator of B-B cell interactions. *J. Exp. Med.*, 173: 137-146, 1991.
31. Pais, S. T., Drilenburg, P., Radaszkiewicz, T., and Mantel-Hurst, E. Adhesion molecules in the dissemination of non-Hodgkin's lymphomas. *Acta Haematol.*, 97: 73-80, 1997.
32. Nitschke, L., Floyd, H., and Crucker, P. R. New functions for the sialic acid-binding adhesion molecule CD22, a member of the growing family of Siglecs. *Scand. J. Immunol.*, 3: 227-234, 2001.

## Antibody-targeted chemotherapy with CMC-544: a CD22-targeted immunoconjugate of calicheamicin for the treatment of B-lymphoid malignancies

John F. DiJoseph, Douglas C. Armellino, Erwin R. Boghaert, Kiran Khandke, Maureen M. Dougher, Latha Sridharan, Arthur Kunz, Philip R. Hamann, Boris Gomperts, Chandrasekhar Lidata, Justin K. Moran, Andrew G. Poplewell, Sue Stephens, Philip Frost, and Nitin K. Damle

Antibody-targeted chemotherapy with *gemtuzumab ozogamicin* (CMA-676, a CD33-targeted immunoconjugate of *N*-acetyl- $\gamma$ -calicheamicin dimethyl hydrazide [CalichDMH], a potent DNA-binding cytotoxic antitumor antibiotic) is a clinically validated therapeutic option for patients with acute myeloid leukemia (AML). Here, we describe the preclinical profile of another immunoconjugate of CalichDMH, CMC-544, targeted to CD22 expressed by B-lymphoid malignancies. CMC-544 comprises a humanized IgG4 anti-CD22 monoclonal antibody (mAb),

G5/44, covalently linked to CalichDMH via an acid-labile 4-(4'-acetylphenoxy) butanoic acid (AcBut) linker. Both CMC-544 and unconjugated G5/44 bound human CD22 with subnanomolar affinity. CMC-544, but not unconjugated G5/44, exerted potent cytotoxicity against CD22<sup>+</sup> B-cell lymphoma (BCL) cell lines (inhibitory concentration of 50%: 6-600 pM CalichDMH). CMC-544 caused a potent inhibition of growth of small but established BCL xenografts leading to cures (therapeutic index > 10). CMC-544 prevented the establishment of BCL xenografts and also

caused regression of large BCLs (> 1.5 g tumor mass). In contrast, unconjugated CalichDMH, unconjugated G5/44, and an isotype-matched control conjugate, CMA-676, were ineffective against these BCL xenografts. Thus, CD22-targeted delivery of CalichDMH is a potent and effective preclinical therapeutic strategy for BCLs. The strong antitumor profile of CMC-544 supports its clinical evaluation as a treatment option for B-lymphoid malignancies. (Blood. 2004;103:1807-1814)

© 2004 by The American Society of Hematology

### Introduction

Antibody-targeted chemotherapy is a therapeutic strategy that involves the use of a cytotoxic agent chemically linked to a monoclonal antibody (mAb) that specifically recognizes a tumor-associated antigen. The mAb specifically delivers the cytotoxic agent to tumor cells that express the tumor-associated antigen. The efficacy of antibody-targeted chemotherapy relies heavily on the specific binding of the targeting mAb-drug conjugate to the tumor antigen and on the internalization of the antigen-mAb complex to ensure focused delivery of the conjugated cytotoxic agent inside tumor cells. Such focused delivery of the cytotoxic agent to tumor cells maximizes its antitumor effect and minimizes its normal tissue exposure, resulting in an improved therapeutic index. The concept of antibody-targeted chemotherapy is not novel and has been explored extensively with significant preclinical success.<sup>1,2</sup> However, it had not been successfully translated into clinical benefit until the recent introduction of *gemtuzumab ozogamicin* (Mylotarg or CMA-676; Wyeth, Philadelphia, PA), the first antibody-targeted chemotherapeutic agent.<sup>3-6</sup> Presently, *gemtuzumab ozogamicin* remains the only antibody-targeted chemotherapeutic agent approved for clinical use in the United States.<sup>7</sup>

*Gemtuzumab ozogamicin* is comprised of a humanized IgG4 anti-CD33 antibody covalently linked to *N*-acetyl- $\gamma$ -calicheamicin dimethyl hydrazide (hereafter referred to as CalichDMH), a derivative of  $\gamma$ -calicheamicin, via an acid-hydrolyzable 4-(4'-acetylphenoxy) butanoic acid (AcBut) linker.<sup>3-8</sup>  $\gamma$ -Calicheamicin is

a potent cytotoxic antitumor antibiotic that binds DNA in the minor groove and undergoes thiol-dependent structural changes in its enediyne moiety to generate a di-radical, which abstracts hydrogens from the phosphodiester backbone of DNA. This causes double-strand DNA breaks resulting in apoptotic cell death.<sup>9-11</sup> *Gemtuzumab ozogamicin* binds CD33 on the surface of myeloid cells and is rapidly internalized into lysosomal vesicles whose acidic pH facilitates hydrolysis of the hydrazone functional group within the AcBut linker liberating CalichDMH.<sup>3-8</sup> *Gemtuzumab ozogamicin* is indicated for the treatment of elderly patients ( $\geq 60$  years of age) with acute myeloid leukemia (AML) in first relapse who are not candidates for other therapies.<sup>3,12</sup> Its effectiveness in patients with AML can be attributed to the preferential delivery of CalichDMH to CD33<sup>+</sup> myeloid leukemic cells resulting in their elimination.<sup>3-8</sup> CD33, the molecular target of *gemtuzumab ozogamicin*, is a cell-surface antigen whose expression is confined to the myeloid lineage. CD33 is expressed by both normal and malignant myeloid cells but is not expressed by nonhematopoietic lineages or stem cell precursors of the myeloid lineage.<sup>13</sup> The clinical effectiveness of *gemtuzumab ozogamicin* provides a strong rationale for applying the antibody-targeted calicheamicin strategy to other malignancies using target antigens with similar lineage-confined expression.

CD22 is an attractive target for antibody-targeted chemotherapy and has been extensively explored as a target for immunotoxin-based therapeutic strategies.<sup>14-16</sup> CD22 is a terminal  $\alpha 2,6$ -linked

From the Departments of Oncology Discovery, Chemical Sciences, Drug Metabolism, and Bioprocess Development, Wyeth Research, Pearl River, NY, and the Department of Research and Development, Cellect, Slough, United Kingdom.

Submitted June 22, 2003; accepted October 1, 2003. Prepublished online as Blood First Edition Paper, November 13, 2003; DOI 10.1182/blood-2003-07-2466.

J.F.D., D.C.A., E.R.B., K.K., M.M.D., L.S., A.K., P.R.H., B.G., C.U., J.K.M., P.F., and N.K.D. are employed by Wyeth Research, whose products (CMA-676 and CMC-544) were studied in the present work. A.G.P. and S.S. are employed by Cellect. Both

CMA-676 and CMC-544 are being jointly developed by Wyeth and Cellect.

Reprints: Nitin K. Damle, Wyeth Research, 200/4604, 401 N Middletown Rd, Pearl River, NY 10965; e-mail: damlen@wyeth.com.

The publication costs of this article were delayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

© 2004 by The American Society of Hematology

sialic acid-binding cellular lectin and a member of the Ig superfamily.<sup>17</sup> CD22 is expressed on the surface of mature B lymphocytes and their malignant counterparts but not on other non-B lineages including hematopoietic stem cells.<sup>17-21</sup> CD22 is rapidly internalized on binding to anti-CD22 mAb.<sup>22,23</sup> These properties make CD22 a suitable molecular target for antibody-targeted calicheamicin therapy for B-lymphoid malignancies. Using a humanized anti-CD22 mAb (G5/44), we have created a CD22-targeted immunoconjugate of CalicheDMH, designated as CMC-544. The present study describes the functional characterization of CMC-544 and provides evidence of its strong preclinical antitumor activity against human B-cell lymphoma (BCL) cell lines, both in vitro and in vivo. This study supports the evaluation of CMC-544 as an effective therapeutic option for patients with BCLs, especially non-Hodgkin lymphoma.

## Materials and methods

### Antibodies

Humanized anti-CD22 (G5/44, IgG4 isotype) was derived from the murine anti-CD22 mAb m5/44 by grafting the complementarity-determining regions (CDRs) plus key framework residues on to human acceptor frameworks<sup>24</sup> at Celltech (Slough, United Kingdom; J.F.D. et al, manuscript in preparation, January 2004). Antibody G5/44 was expressed in Chinese hamster ovary cells and purified at Wyeth Biopharma (Andover, MA). G5/44 was covalently linked to CalicheDMH via an acid-labile AcBut linker as described<sup>4</sup> and the resulting conjugate was designated as CMC-544. Figure 1 shows a structural representation of CMC-544, which has an average loading of 65 to 80  $\mu$ g CalicheDMH/mg antibody protein (5-7 moles CalicheDMH/mol antibody) and less than 10% of the antibody protein in CMC-544 is unconjugated. This conjugate preparation contains less than 1  $\mu$ g unconjugated CalicheDMH/mg conjugated antibody. CMA-676 (gemtuzumab ozogamicin) is a conjugate of humanized IgG4 anti-human CD33 mAb, hP67.6, covalently linked to CalicheDMH via the AcBut linker.<sup>2-6</sup>

All conjugates were determined to be endotoxin free ( $< 5.0$  endotoxin units/mL [EU/mL]) by a modified limulus amoebocyte assay (BioWittaker, Walkersville, MD). Rituximab (Rituxan; Genentech, South San Francisco, CA),<sup>25</sup> a chimeric IgG1 anti-CD20 mAb, was obtained from Med World Pharmacy (Chestnut Ridge, NY). Purified human IgG4 and IgG1 were obtained from Sigma Chemical (St Louis, MO). A fusion protein consisting of the extracellular domain of human CD22 genetically fused to the hinge-CH2-CH3 Fc region of murine IgG1 (CD22mFc) was expressed in NS0 cells and purified at Celltech R&D. Fluorescein-conjugated affinity-purified polyclonal goat antihuman IgG (heavy and light chain specific) antibody was obtained from Zymed (South San Francisco, CA).

### Cell lines

Burkitt lymphoma cell lines Ramos (CRL-1923), Raji (CCL-86), Daudi (CCL-213), a non-Hodgkin lymphoma cell line RL (CRL-2261), and a

myeloid leukemic cell line HL-60 (CCL-240) were all obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cell lines were determined to be mycoplasma free by a polymerase chain reaction mycoplasma detection assay (ATCC). The cell lines Ramos, Raji, Daudi, and RL were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 1 mM sodium pyruvate, 0.2% glucose, penicillin G sodium 100 U/mL, and streptomycin sulfate 100  $\mu$ g/mL. HL-60 cells were propagated and maintained in Iscove Dulbecco modified Eagle medium (DMEM) plus 20% FBS, 0.02% glucose, penicillin G sodium 100 U/mL, and streptomycin sulfate 100  $\mu$ g/mL.

### Mice

Female, athymic BALB/c nu/nu (nude) mice (18-23 g) were obtained from Charles River (Wilmington, MA). All mice were provided with food and water ad libitum throughout the studies. All experimental procedures involving mice were carried out in a laminar flow hood and were approved by the Wyeth Pearl River Animal Care and Use Committee according to the established guidelines.

### Biosensor analysis

Biosensor analyses were carried out using a BiAcore 2000 (BiAcore, Uppsala, Sweden). CD22mFc was covalently immobilized on the N-hydroxysuccinimide-activated carboxymethyl dextran-coated biosensor chip (CM5) using a standard amine-coupling chemistry at a protein density of approximately 2000 resonance units. Samples of CMC-544 or G5/44 were diluted in the HBS buffer (10 mM HEPES, pH 7.4, containing 150 mM NaCl, 3 mM EDTA [ethylenediaminetetraacetic acid], and 0.005% polysorbate 20 [v/v]) and injected in the concentration range of 1 to 100 nM over the CD22mFc-coated biosensor chip surface at a flow rate of 30  $\mu$ L/min for 3 minutes to allow binding. After the binding phase, dissociation of the bound antibody was monitored by washing the chip with the HBS buffer over a 15-minute period. The antigenic surface was regenerated by washing the biosensor chip with 15  $\mu$ L of the regeneration buffer (10 mM NaOH and 200 mM NaCl) for 30 seconds, followed by a stabilization time of 2 minutes before the next cycle. Kinetic constants were calculated by nonlinear least square regression analysis using a 1:1 Langmuir binding curve fitting model and BIAevaluation program (version 3.0, BiAcore).<sup>26</sup>

### Cytotoxicity assays

Various BCL cells were cultured in the presence of various concentrations of unconjugated CalicheDMH, unconjugated antibody (G5/44), CMC-544, or CMA-676 for 96 hours after which cell viability was measured by propidium iodide exclusion and analyzed by flow cytometry using a Becton Dickinson FACSort (Becton Dickinson, Franklin Lakes, NJ). Red fluorescent intensity (emission at 617 nm in the FL2 channel) of the cells excited at 488 nm was measured. The regions for viable cells were also set using both the forward light scatter and right-angle light scatter properties of the cells. The loss of viability was determined by the loss of cells from within the gated region defining viable cells. The average number of viable cells per 6 replicate cultures was calculated. The percent survival of BCL cells in these cultures was calculated using the following equation: percent survival =  $100 \times (\text{no. of viable cells in treated cultures} / \text{no. of viable cells in control cultures})$ . The inhibitory concentration of 50% (IC<sub>50</sub> with 95% confidence interval [CI]) was calculated by logistic nonlinear regression and is reported as the amount of CalicheDMH equivalents from each treatment group that causes 50% loss of cell viability.

### Subcutaneous xenografts

Female, athymic nude mice were exposed to total body irradiation (4 Gy [400 rad]) to further suppress their residual immune system and facilitate the establishment of BCL xenografts. Three days later, the irradiated mice were injected subcutaneously with  $1 \times 10^7$  Ramos or RL cells in Matrigel (Collaborative Biomedical Products, Bedford, MA, diluted 1:1 in RPMI 1640 medium) in the dorsal, left flank. When the tumors reached the mass of 0.3 to 0.4 g, the tumors were staged to ensure uniformity of the tumor

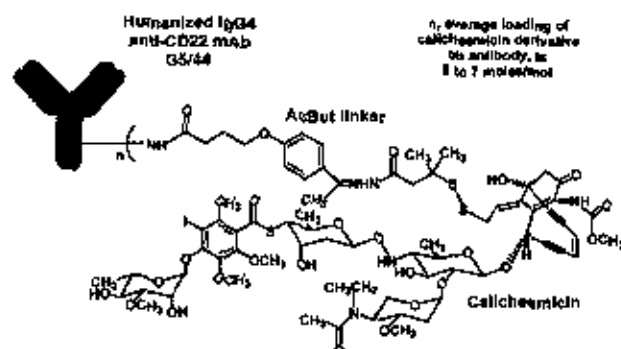


Figure 1. Structure of CMC-544, a CD22-targeted immunoconjugate of CalicheDMH.

mass between various treatment groups ( $n = 7-9$  mice/group) prior to the administration of therapy. Conjugated CalichDMH (CMC-544 or CMA-676), unconjugated CalichDMH, or unconjugated antibody (G5/44) was administered intraperitoneally in sterile saline (0.2 mL/mouse) on day 1 and the same treatment was repeated twice 4 days apart. Doses of CMC-544 and CMA-676 were based on the quantity of CalichDMH. In additional studies, the effect of CMC-544 or G5/44 on the development of the BCLs was also examined. In these studies, treatments were administered 3 days after the BCLs were injected subcutaneously. Tumors were measured at least once a week and their mass was defined as tumor mass (g) =  $0.5 \times (\text{tumor width}^2)(\text{tumor length})$ . Mean tumor mass ( $\pm$  SEM) for each treatment group was calculated and compared to the vehicle-treated group for statistical significance using a one-sided  $t$  test, with the error term for the  $t$  test based on the pooled variance across all treatment groups. Tumor mass values for each treatment group were recorded up to 100 days after the initiation of treatment or until either tumor-bearing mice died or the tumors grew to 15% of the body weight at which time these mice were humanely killed according to institutional regulations. The number of tumor-free mice at the end of each study for each treatment group was also recorded. The minimum efficacious dose (MED) of CMC-544 was the lowest dose of CMC-544 that caused a statistically significant inhibition of tumor growth ( $P < .05$ ) at any given time point compared to the vehicle-treated group.

To determine the maximum nonlethal dose (MND) of CMC-544, nonirradiated, nontumor-bearing mice ( $n = 10$ ) were administered intraperitoneally increasing doses of CMC-544 as 3 doses, 1 every 4 days, and monitored for 50 days. The MND was the highest dose of CMC-544 at which 100% of the treated mice survived the observation period. The therapeutic index (TI) was calculated as the ratio of the MND/MED. The minimum curative dose (MCD) was the lowest dose of CMC-544 at which no residual tumor mass could be detected and the mice remained tumor-free for at least 100 days after initiation of the treatment. The curative index was calculated as the ratio of MND/MCD. ED<sub>50</sub> is the dose of CMC-544 (3 doses, 1 every 4 days) at which 50% inhibition of average tumor growth is observed relative to the vehicle-treated average tumor growth (with 95% CIs) and was estimated using inverse prediction after fitting a logistic model to relative tumor growth data from 3 independent experiments using RL BCL xenografts.

#### Pharmacokinetics of CMC-544 in tumor-bearing and nontumor-bearing mice

RL BCL-bearing nude mice (subcutaneous tumors) and nontumor-bearing nude mice ( $n = 52$ /group) were given a single intraperitoneal dose of CMC-544 (160  $\mu$ g conjugated CalichDMH/kg) at time 0. Blood samples were collected terminally by cardiac puncture under CO<sub>2</sub> anesthesia from these mice ( $n = 4$  mice/time point) using a nonserial bleeding design at 0.1, 0.5, 1, 4, 8, 24, 48, 72, 96, 120, 168, 240, and 336 hours after dosing. Serum from these blood samples was collected and stored frozen at  $-70^\circ\text{C}$ . Serum concentrations of CMC-544 were quantified using a modification of an enzyme-linked immunosorbent assay (ELISA) originally developed for the pharmacokinetic measurements of CMA-676.<sup>27</sup> A soluble form of the CD22 protein, CD22mFc, was immobilized on microtiter plates to capture the G5/44 antibody portion within CMC-544. The CalichDMH portion of the molecule was then recognized with a rabbit anticalicemicin antibody. A goat antirabbit antibody conjugated to horseradish peroxidase was used to detect the bound rabbit antibody using 3,3',5,5'-tetramethylbenzidine

(TMB) as a substrate for colorimetric readout. CMC-544 diluted in nude mouse serum was used as a standard in these determinations. The pharmacokinetic parameters for each dose group were estimated using the pharmacokinetic and statistical analysis software, IBIS (version 1.5.0) that runs under SAS (version 6.12). Calculations were performed using a model-independent approach as described by Gibaldi and Perrier.<sup>28</sup> Briefly,  $C_{max}$  and  $t_{max}$  values were taken directly from the observed mean data. The area under the serum concentration versus time curve (AUC) over the sampling period of 336 hours and its SE were calculated using a method described by French and Powers<sup>29</sup> that is based on a rearrangement of the linear trapezoidal rule.

## Results

### Binding of CMC-544 to CD22

The binding of CMC-544 to human CD22 was evaluated by surface plasmon resonance analysis using CD22mFc covalently immobilized on a biosensor chip. The results of kinetic analyses of the binding of CMC-544 and G5/44 to CD22mFc are shown in Figure 2. The data were fitted globally to a 1:1 Langmuir binding model with compensation for mass transfer. Both CMC-544 and unconjugated G5/44 bound CD22 with a similar affinity (CMC-544/CD22  $K_D = 200$  pM; G5/44/CD22  $K_D = 235$  pM). Conjugation to CalichDMH did not have an impact on the ability of G5/44 to bind CD22mFc effectively. The binding of CMC-544 and G5/44 to CD22 expressed on the surface of human B-lymphoma cells was further confirmed by flow cytometry. CMC-544 specifically recognizes CD22 on human B cells but not on murine, rat, canine, porcine, or primate (cynomolgus and rhesus) B cells (data not shown).

### In vitro effects of CMC-544 against human B-lymphoma cells

The effect of CMC-544 on the in vitro growth of various CD22 B-lymphoma cell lines was determined. CD33-targeted CMA-676 was used as an isotype-matched nonbinding control conjugate to explore antigen-nonspecific effects of the conjugates. CalichDMH was also included for comparison in these studies. The use of unconjugated CalichDMH indicated sensitivity of each cell line to this drug. Table 1 shows results of the cytotoxic effect (IC<sub>50</sub>) based on the CalichDMH equivalents defined as the concentration of conjugated CalichDMH that causes 50% reduction in the cell number compared to untreated controls of CMC-544 and CMA-676. CMC-544 was more potent (1.5- to 39-fold) against CD22<sup>+</sup> B-lymphoma cells (Ramos, Raji, and RL) than unconjugated CalichDMH. The cytotoxic effect of the isotype-matched control conjugate (CMA-676) against CD22<sup>+</sup> B-lymphoma cells was less than that of unconjugated CalichDMH. Unconjugated G5/44 mAb up to 1  $\mu$ M (antibody protein) had no effect on the in vitro growth of any of the B-lymphoma cell lines studied. Against the CD22<sup>+</sup>CD33<sup>+</sup> leukemic cell line HL-60, the relative potencies

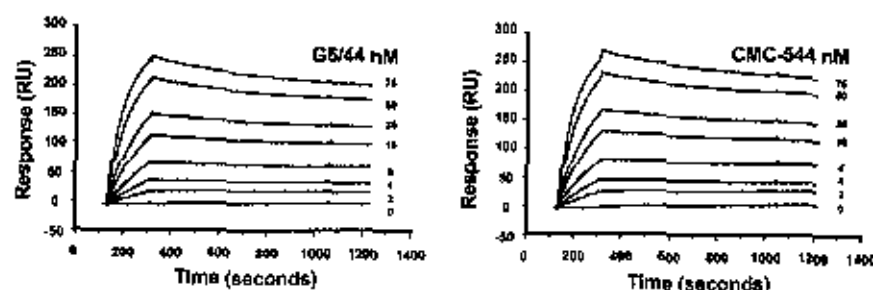


Figure 2. Sensorgrams from biosensor analysis of the interaction between G5/44 or CMC-544 and immobilized CD22mFc. G5/44 (left) or CMC-544 (right) at indicated protein concentrations was injected over CD22mFc immobilized onto the biosensor chip surface. Curves were fitted to a 1:1 Langmuir binding model with allowance for mass transfer effects.

**Table 1. Cytotoxic activity of calicheamicin immunconjugates against human B-lymphoma cell lines in vitro**

| Human tumor cell line   | IC <sub>50</sub> = pM (CalichDMH equivalents) |                       |                                   |
|-------------------------|-----------------------------------------------|-----------------------|-----------------------------------|
|                         | Conjugated CalichDMH                          |                       | Unconjugated untargeted CalichDMH |
|                         | CD22-targeted CMC-544                         | CD33-targeted CMA-676 |                                   |
| CD22 <sup>+</sup> RL    | 8                                             | 827                   | 234                               |
| CD22 <sup>+</sup> Daudi | 21                                            | 1886                  | 280                               |
| CD22 <sup>+</sup> Raji  | 300                                           | 2800                  | 460                               |
| CD22 <sup>+</sup> Ramos | 200                                           | 1110                  | 700                               |
| CD33 <sup>+</sup> HL-60 | 2500                                          | < 200                 | 750                               |

Human B-lymphoma cells were cultured for 96 hours in the presence of various concentrations of CMC-544, CMA-676, or unconjugated CalichDMH after which the viable cell number in each culture was enumerated by their exclusion of propidium iodide and detected by flow cytometry. Unconjugated anti-CD22 mAb G5/44 up to 1  $\mu$ M had no effect on the growth of these B-cell lines. Results are IC<sub>50</sub> values expressed in pM CalichDMH equivalents.

of CD22-targeted CMC-544 and CD33-targeted CMA-676 were reversed. CMA-676 was at least 12-fold more potent than CMC-544 against HL-60 cells. The antigen-nonspecific effects of CMC-544 and CMA-676 can be explained by the hydrolytic release of CalichDMH from its conjugated state by the acidification of the culture medium of actively cycling cells. These results demonstrate that CMC-544 exerts CD22-specific cytotoxic effect with subnanomolar potency against CD22<sup>+</sup> B-lymphoma cells. Furthermore, the observed greater cytotoxic potency of CMC-544 over unconjugated CalichDMH is indicative of the efficiency of CD22-mediated intracellular delivery of CalichDMH over its untargeted transport through the cell membrane.

#### Antitumor efficacy of CMC-544 against established B-lymphoma xenografts

CMC-544 was evaluated for its antitumor activity against established SC xenografts of CD22<sup>+</sup> Ramos or RL human BCLs. The tumor-bearing mice were staged to an average tumor mass of 300 mg (established, small subcutaneous tumors). CMC-544 was administered at 10 to 320  $\mu$ g conjugated CalichDMH/kg body weight (0.15–5 mg/kg of conjugated antibody protein) intraperitoneally on days 1, 5, and 9 (3 doses, 1 every 4 days). CMA-676 was similarly administered as a negative control conjugate. This dosing schedule has been shown to be effective for immunoconjugates of CalichDMH in a number of preclinical tumor models.<sup>7,8,30</sup> CMC-544 inhibited the subcutaneous growth of Ramos BCL in a dose-dependent manner (Figure 3). The lowest dose of CMC-544 causing significant ( $P < .05$ ) growth inhibition (MED) of Ramos BCL xenografts was 10  $\mu$ g conjugated CalichDMH/kg. CMC-544, administered intraperitoneally as 3 doses, 1 every 4 days at 160  $\mu$ g conjugated CalichDMH/kg, caused complete regression of Ramos BCL xenografts and 6 of 7 mice in this treatment group remained tumor-free up to 100 days and were considered cured. In a separate experiment (data not shown), CMC-544 at the dose of 120  $\mu$ g conjugated CalichDMH/kg (MCD) also cured Ramos xenografted mice. In contrast, CMA-676 (isotype-matched nonbinding control conjugate), at 160  $\mu$ g/kg (administered intraperitoneally as 3 doses, 1 every 4 days) had no significant effect on the growth of Ramos BCL xenografts.

The ability of CMC-544 to inhibit the establishment of developing Ramos BCL xenografts was examined. Ramos cells were injected subcutaneously and 3 days later intraperitoneal treatment (3 doses, 1 every 4 days) with CMC-544 was initiated. CMC-544 completely suppressed the establishment of the developing Ramos BCL (Figure 4A). The effect of CMC-544 was also studied with

large (almost 10% of the body weight) Ramos BCL xenografts. Ramos xenografts were staged at 2 g tumor mass after which treatment with CMC-544 was initiated. CMA-676 was again used as a negative control conjugate. In the CMA-676 treatment group, the tumors grew rapidly and within less than 2 weeks after initiation of therapy, these mice had to be humanely killed. In contrast, similar treatment with CMC-544 caused almost complete regression of lymphoma xenografts (Figure 4B). Monitoring these tumor-free mice for up to 50 days did not demonstrate any regrowth of the Ramos BCL. Thus, CMC-544 not only inhibited the establishment of BCL xenografts but also caused precipitous regression of large BCL xenografts.

A similar evaluation of the antitumor effect of CMC-544 was carried out using the RL BCL xenograft model. CMC-544, in a dose-dependent manner, caused RL xenografts to regress within 3 weeks. In contrast, neither unconjugated CalichDMH nor a mixture of unconjugated CalichDMH and unconjugated G5/44 had any effect on the growth of RL BCL xenografts. The MED of CMC-544 in the RL lymphoma model was 20  $\mu$ g conjugated CalichDMH/kg (Figure 5). Regression analysis of the dose response of CMC-544 from this and additional studies not shown in Figure 5 indicated the ED<sub>50</sub> to be 53  $\mu$ g conjugated CalichDMH/kg (95% CI, 19.6–137.6). The MCD of CMC-544 in the RL BCL model was 160  $\mu$ g/kg. CMC-544, administered as 3 doses, 1 every 4 days via either the intraperitoneal or intravenous route, had almost identical antitumor activity regardless of the dose of the conjugate (data not shown). Taken together, these results obtained with 2 distinct BCL xenograft models clearly demonstrate the ability of CMC-544 to cause regression of developing as well as established BCL tumors.

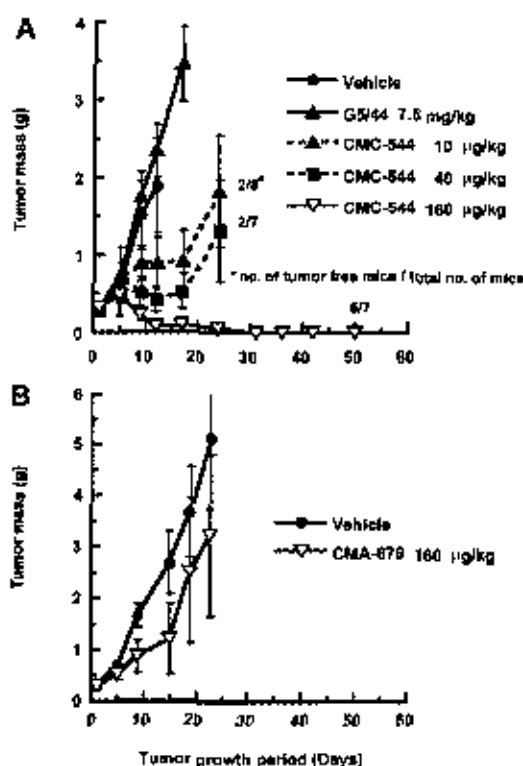


**Figure 3. Effect of CMC-544 or CMA-676 on small Ramos BCL xenografts.** (A) Effect of CMC-544 on the growth of small but established Ramos BCL xenografts is shown. The effect of an isotype-matched control conjugate, CMA-676, was also evaluated in the same tumor model (B). G5/44 dose is shown in antibody protein equivalents and CMC-544 dose is shown in CalichDMH equivalents. The dose of 7.5 mg/kg G5/44 is equivalent to the dose of antibody protein in 160  $\mu$ g/kg of CMC-544. Results are presented as mean tumor mass  $\pm$  SEM.

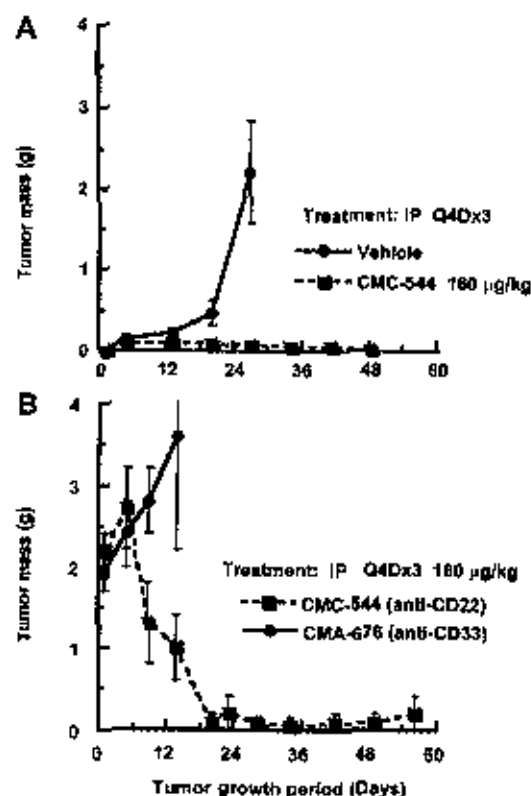


**Figure 4.** Effect of CMC-544 on developing or large Ramos BCL xenografts. Effect of CMC-544 on developing (A) or large established Ramos BCL xenografts (B). CMA-676 was used as a nonblinding control conjugate. Doses are shown in CalicheDMH equivalents. Results are presented as mean tumor mass  $\pm$  SEM.

The effect of unconjugated anti-CD22 mAb G5/44 on the growth of developing or established RL BCL was also investigated. Administration of G5/44 at a dose as high as 20 mg/kg intraperitoneally did not influence the growth of developing (Figure 6A) or established (Figure 6B) RL BCL xenografts. The dose of 20 mg/kg of G5/44 was selected based on the ability of anti-CD20 rituximab

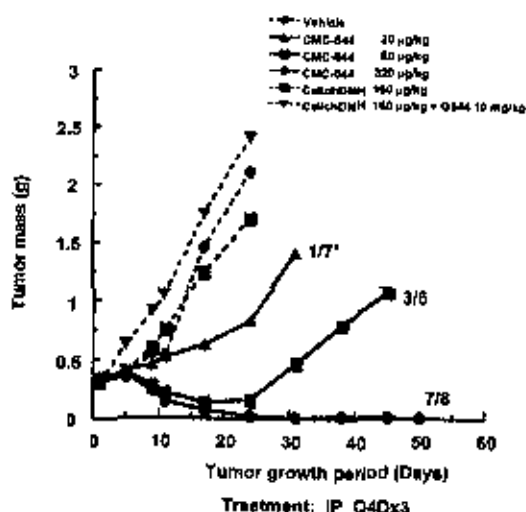


**Figure 5.** Effect of CMC-544, unconjugated CalicheDMH, or an admixture unconjugated anti-CD22 mAb G5/44 and CalicheDMH on the growth of small but established RL BCL xenografts. CMC-544 dose is shown in CalicheDMH equivalents. Unconjugated G5/44 dose is in antibody protein equivalents. \*Number of tumor-free mice/number of total mice.

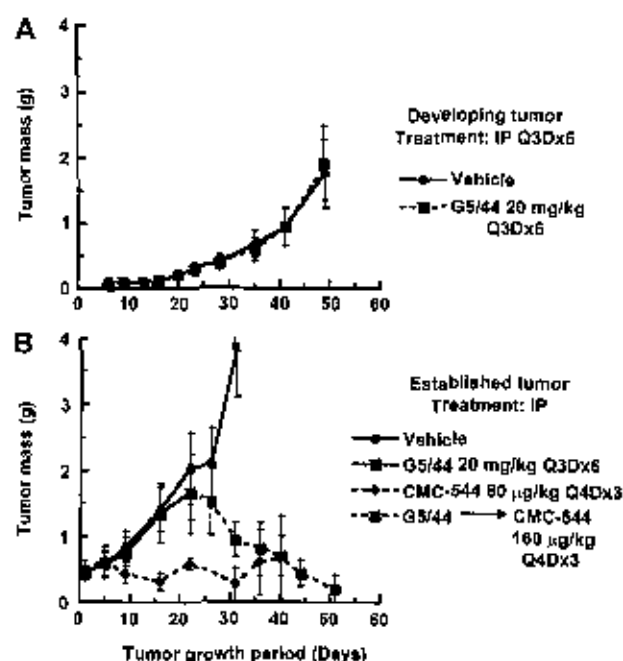


**Figure 6.** Effect of unconjugated anti-CD22 mAb G5/44 and CMC-544 on RL BCL xenografts. Effect of unconjugated anti-CD22 mAb G5/44 on developing (A) or small but established (B) RL BCL xenografts. In addition, CMC-544 was also administered as 3 doses, 1 every 4 days treatments starting day 23 to established RL BCL xenograft-bearing mice that had been pretreated with unconjugated G5/44 over a period of 21 days (B). CMC-544 was also administered on day 1, then again on days 5 and 9, as a positive control. G5/44 dose is shown in antibody protein equivalents and CMC-544 dose is shown in CalicheDMH equivalents. Error bars show standard deviation (SD).

to inhibit the establishment of RL BCL at this dose (data not shown). In contrast, CMC-544, administered intraperitoneally at a suboptimal dose of 80 µg conjugated CalicheDMH/kg as 3 doses, 1 every 4 days, suppressed the growth of small RL xenografts. Large RL BCL xenografts, which had grown despite the high-dose treatment with unconjugated G5/44, were further treated with CMC-544 and regressed in response to the CMC-544 treatment (Figure 6B). Thus in the absence of CalicheDMH, the unconjugated anti-CD22 mAb G5/44 had no impact on the growth of BCL xenografts and these G5/44-treated BCLs were still susceptible to CMC-544.

#### Pharmacokinetics of CMC-544 in nude mice

To determine the serum levels of CMC-544 that were associated with the antitumor effects, tumor-bearing (subcutaneous RL B lymphoma) or nontumor-bearing nude mice received a single intraperitoneal dose of CMC-544 at 160 µg conjugated CalicheDMH/kg and serum samples from these mice were assayed for the presence of CMC-544 using an enzyme-linked immunoassay. The immunoassay was specifically designed and validated to detect antibody conjugates of CalicheDMH.<sup>27</sup> As shown in Figure 7, the mean ( $\pm$  SE)  $C_{max}$  values for nontumor-bearing and RL BCL-bearing nude mice, respectively, were  $2.6 \pm 0.1$  and  $2.4 \pm 0.2$  µg/mL (CalicheDMH equivalents of CMC-544), the  $t_{1/2}$  were 34.2 and 35 hours, and the corresponding mean  $AUC_{0-\infty}$  values were 145 and 93 µg/h/mL in nontumor-bearing and RL BCL-bearing mice, respectively. The difference between the  $AUC_{0-\infty}$  values for nontumor-bearing mice and RL BCL-bearing mice was statistically significant ( $P < .05$ ). The single dose of CMC-544 administered in this study prevented the growth of the existing RL BCL mass for 9 days after which tumor measurements were not taken. The lower systemic exposure ( $AUC_{0-\infty}$ ) of CMC-544 in tumor-bearing mice

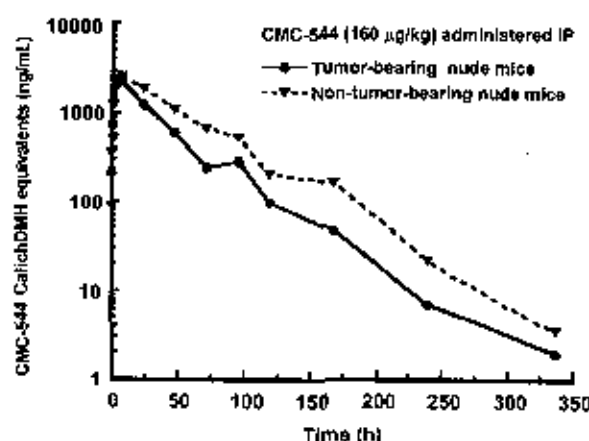


Figure 7. Pharmacokinetics of CMC-544 in nude mice. CMC-544 was administered intraperitoneally at 160  $\mu$ g/kg to tumor-bearing (RL BCL) and non-tumor-bearing nude mice. Serum was collected from these mice at different time intervals ( $n = 3$  mice/time point) and assessed for the presence of CMC-544 as described in "Materials and methods." CMC-544 is expressed as CalichDMH equivalents.

than that in nontumor-bearing mice is consistent with the uptake of CMC-544 by the tumor.

#### Determination of MND of CMC-544 in BALB/c nude mice

To determine the MND of CMC-544, nontumor-bearing nude mice (10 animals/group) were administered CMC-544 (80-640  $\mu$ g conjugated CalichDMH/kg intraperitoneally as 3 doses, 1 every 4 days) and their survival was monitored daily for up to 50 days. All mice receiving CMC-544 at doses of 240  $\mu$ g or less of conjugated CalichDMH/kg survived the entire period of evaluation (Table 2). However, at the next higher dose of 320  $\mu$ g/kg of CMC-544, only 4 of 10 mice were alive by day 50. Thus, the dose of 240  $\mu$ g/kg of CMC-544 was regarded as the MND of CMC-544. It is interesting to note that the dose of 320  $\mu$ g/kg, which was the LD<sub>50</sub> in nontumor-bearing mice, was curative and nonlethal in RL B-lymphoma-bearing mice. This observation is consistent with the reduced systemic exposure of tumor-bearing mice versus nontumor-bearing mice as noted. CMC-544 demonstrates strong antitumor activity against BCL xenografts with therapeutic indices (MND/MED) of 24 and 12 in Ramos and RL xenograft models, respectively, and curative indices (MND/MCD) of 2 and 1.5 in Ramos and RL models, respectively.

Collectively, these results suggest that CMC-544 not only prevents the establishment of subcutaneous BCL xenografts but also causes regression of established BCL xenografts at doses lower than its MND dose.

## Discussion

Antibody-targeted chemotherapy is an emerging therapeutic strategy that preferentially delivers the cytotoxic agent to tumor cells and minimizes the exposure of normal tissues resulting in an improved therapeutic index.<sup>31</sup> CMC-544 represents a new therapeutic opportunity for CD22-targeted delivery of calicheamicin to various B-cell malignancies. CD22 was chosen as a target for conjugate delivery for several reasons. CD22 is preferentially expressed on both normal and malignant cells of the mature B-lymphocyte lineage.<sup>20,21</sup> It is not expressed on non-B-lymphoid cells, myeloid cells, hematopoietic stem cells, hematopoietic precursors of B lymphocytes, or any other nonhematopoietic

lineage. CD22-targeted chemotherapy is not expected to affect specifically any tissue nor expressing CD22 and it should not inhibit the generation of new B cells from their hematopoietic progenitors. Moreover, CD22 is one of the better internalizing molecules expressed on the surface of mature and malignant B cells<sup>22,23</sup> and is not routinely shed into the extracellular environment. The preclinical antitumor activity of a number of CD22-targeted immunotoxins prepared with various xenotoxins has been reviewed.<sup>14-16</sup> Of interest in this context is a recent clinical study by Kreitman et al in which a recombinant immunotoxin (BL22 targeted to CD22) was shown to cause complete remission in 68% of patients with chemorefractory hairy cell leukemia,<sup>32</sup> thus further validating the choice of CD22 as a targeting antigen.

CMC-544 bound CD22 with subnanomolar affinity and exhibited a potent, dose-dependent cytotoxicity against CD22<sup>+</sup> B-lymphoma cells. The CD22-mediated intracellular delivery of CalichDMH was more effective in exerting cytotoxic activity than the untargeted uptake of CalichDMH. This enhanced potency can be attributed to the efficient internalization and subsequent intracellular delivery of the CD22-bound CMC-544. In contrast, unconjugated G5/44 antibody had no effect on the growth of various BCLs, both in vitro and in vivo. CMC-544 caused a dose-dependent regression of BCL xenografts grown as subcutaneous solid tumors in athymic nude mice. It prevented the development of BCL growth and was also effective in causing regression of preexisting small and large established BCLs. The growth of established BCLs was inhibited by CMC-544 at a dose less than one tenth the MND (240  $\mu$ g/kg) and cured tumor-bearing mice of the tumor at half the MND. The antitumor effect of CMC-544 requires that both the targeting antibody and CalichDMH are chemically linked to each other. Neither unconjugated G5/44, unconjugated CalichDMH, nor their admixture had any effect on the BCL growth in vivo. Not surprisingly, in the absence of the targeting moiety, unconjugated CalichDMH lacked antitumor activity at the dose evaluated, further illustrating the advantage of tumor-targeted therapy. Consequently, we conclude that the effectiveness of CMC-544 observed in vivo in this study was a direct result of the specific and targeted delivery of CalichDMH to tumor cells.

Unconjugated G5/44, an IgG4 isotype anti-CD22 antibody, did not inhibit the establishment of BCL xenografts nor their subsequent growth. This lack of effect of G5/44 in vivo is consistent with its inability to inhibit the growth of BCL in vitro. Human IgG4 antibodies fix complement poorly, are poor binders to Fc receptors, and, thus, cannot support complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC). The lack of antitumor effect of G5/44 in the BCL xenograft models was not due to the inability of murine FcR to mediate ADCC or murine

Table 2. Maximum tolerated nonlethal dose of CMC-544 in nude mice

| Dose of CMC-544,<br>$\mu$ g conjugated CalichDMH/kg | Survival of<br>nude mice, % |
|-----------------------------------------------------|-----------------------------|
| 0                                                   | 100                         |
| 80                                                  | 100                         |
| 160                                                 | 100                         |
| 240                                                 | 100                         |
| 320                                                 | 40                          |
| 400                                                 | 30                          |
| 480                                                 | 0                           |

CMC-544 was administered intraperitoneally as 3 doses, 1 every 4 days at various doses to nontumor-bearing nude mice (10 mice/group) after which their survival was monitored for up to 50 days. Maximum nonlethal dose (MND) of CMC-544 was the highest dose at which all mice treated with CMC-544 survived after 50 days.

complement to mediate CDC with human(ized) IgG antibodies nor was the lack of activity by G5/44 due to the resistance of the B-lymphoma cells toward ADCC or CDC because rituximab, an anti-CD20 chimeric mAb, at a dose of 20 mg/kg inhibited the establishment of RL BCL xenografts in nude mice (data not shown). Rimiximab is a chimeric mAb with human IgG1 constant region that can mediate both ADCC and CDC and the antitumor activity of rituximab is consistent with these capabilities.<sup>33,34</sup> Another CD22-targeted humanized IgG1 antibody, epratuzumab, has also been shown to mediate both ADCC and CDC.<sup>35</sup> The clinical activity of both rituximab and epratuzumab is consistent with their ability to mediate both these activities.<sup>31,35</sup> Because the targeting antibody in CMC-544 lacks these effector capabilities, CMC-544 should not be regarded as an immunotherapeutic agent but rather a CD22-targeted chemotherapeutic agent.

When serum CMC-544 levels were analyzed, the  $C_{max}$  of CMC-544 was approximately 2.5  $\mu$ g CalichDMH/mL serum (1.67  $\mu$ M) in both tumor- and nontumor-bearing mice. The  $IC_{50}$  values calculated in vitro for CMC-544's cytotoxic activity against a series of B-lymphoma cells ranged from 0.009 to 0.9 ng CalichDMH equivalents/mL (6–600 pM). Therefore, the maximum serum level of CMC-544 (after a single intraperitoneal dose) was substantially higher than that needed to produce cytotoxic effects in vitro. Given the systemic  $t_{1/2}$  of 35 hours for CMC-544, the dosing regimen of 3 doses, 1 every 4 days ensures that a constant and efficacious systemic exposure of CMC-544 is maintained over a 2-week period resulting in tumor regression. The  $AUC_{0-\infty}$  values of CMC-544 in tumor-bearing mice were 37% lower ( $P < .05$ ) than in nontumor-bearing mice suggesting that the conjugate can be targeted to and absorbed by the tumor, thereby decreasing serum levels of CMC-544 in tumor-bearing mice. The  $t_{1/2}$  of CMC-544 in tumor-bearing and nontumor-bearing nude mice was similar.

A number of B cell-targeted immunotoxins have been evaluated as a therapy for BCL, both preclinically and clinically<sup>14–16,32,36</sup> and, although objective clinical responses were observed, dose-

limiting toxicities of these agents such as vascular leak syndrome have precluded their broader clinical use.<sup>14–16</sup> An additional limiting feature of the immunotoxins is their immunogenicity in man. Patients treated with these agents developed antibodies that negatively affected the pharmacokinetics of these agents and may have neutralized their antitumor activity.<sup>34,35</sup> Clinical experience with gemtuzumab ozogamicin has demonstrated that it is relatively nonimmunogenic in humans. No antibody response was detected to either the targeting antibody nor the CalichDMH-linker component of gemtuzumab ozogamicin in phase 2 clinical trials involving more than 200 patients.<sup>3</sup> Based on the clinical experience with gemtuzumab ozogamicin, CMC-544 is likely to be poorly immunogenic in man.

In summary, CMC-544, as a potent antibody-targeted chemotherapeutic agent, shows promising activity in preclinical models of BCL and may provide a strong antitumor therapeutic advantage in humans. CMC-544 should be regarded as a targeted chemotherapeutic agent and not an immunotherapeutic agent, and its safety profile should be compared with that of chemotherapeutic agents currently used in the treatment of B-cell malignancies. Although some toxicity may be observed with CMC-544 due to the potency of CalichDMH, its targeted delivery is expected to result in less severe toxicity than the nontargeted systemic delivery of currently used cytotoxic combination chemotherapy. How extensively this agent can be used in a clinical setting will be guided largely by its clinical safety and efficacy profile. CMC-544 is currently being evaluated as a targeted chemotherapeutic for patients with non-Hodgkin B-cell lymphoma in a multicenter phase I clinical trial.

## Acknowledgments

The authors would like to thank Jorge Quiroz and Fred Immermann for statistical analyses.

## References

- Trail P, Bianchi A. Monoclonal antibody drug conjugates in the treatment of cancer. *Current Opin Immunol*. 1999;11:584–588.
- Dubowchik G, Walker M. Receptor-mediated and enzyme-dependent targeting of cytotoxic anticancer drugs. *Pharmacol Ther*. 1999;83:67–123.
- Bross PF, Baltz J, Chen G, et al. Gemtuzumab ozogamicin: approval summary. *Gemtuzumab ozogamicin in relapsed acute myeloid leukemia*. *Clin Cancer Res*. 2001;7:1490–1496.
- Berger M, Leopold L, Dowell J, Kortz-Bradley J, Shuman M. Licensure of gemtuzumab ozogamicin for the treatment of selected patients 60 years of age or older with acute myeloid leukemia in first relapse. *Invest New Drugs*. 2002;20:395–406.
- Slevers E, Larson R, Stadmaier E, et al. Efficacy and safety of gemtuzumab ozogamicin in patients with CD33-positive acute myeloid leukemia in first relapse. *J Clin Oncol*. 2001;19:3244–3254.
- Larson R, Boogaerts M, Esley E, et al. Antibody-targeted chemotherapy of older patients with acute myeloid leukemia in first relapse using Mylotarg (gemtuzumab ozogamicin). *Leukemia*. 2002;16:1627–1636.
- Hamann P, Hinman L, Beyer C, et al. An anti-CD33 antibody-calicheamicin conjugate for treatment of acute myeloid leukemia. Choice of linker. *Bioconjug Chem*. 2002;13:40–46.
- Hamann P, Hinman L, Hollander I, et al. Gemtuzumab ozogamicin, a potent and selective anti-CD33 antibody-calicheamicin conjugate for treatment of acute myeloid leukemia. *Bioconjug Chem*. 2002;13:47–53.
- Lee M, Dunne T, Chang C, et al. Calicheamicins, a novel family of antitumor antibiotics. 4: structural elucidation of calicheamicins. *J Am Chem Soc*. 1992;114:965–967.
- Zein N, Sinha A, McGahren W, Ellestad G. Calicheamicin  $\gamma_1$ , an antitumor antibiotic that cleaves double-stranded DNA site specifically. *Science*. 1988;240:1195–1201.
- Thorson J, Slevers E, Ahlert J, et al. Understanding and exploiting Nature's chemical arsenal: the past, present and future of calicheamicin research. *Current Pharm Des*. 2000;6:1841–1879.
- FDA. Mylotarg label. Available at: <http://www.fda.gov/cder/foi/label/2000/211741b1.pdf>. Accessed on June 1, 2003.
- Andrews R, Singer J, Barnstein I. Precursors of colony-forming cells in humans can be distinguished from colony-forming cells by expression of the CD33 and CD34 antigens and high scatter properties. *J Exp Med*. 1989;169:1721–1731.
- Kreitman RJ. Toxin-labeled monoclonal antibodies. *Curr Pharm Biotechnol*. 2001;2:313–325.
- Pastan I, Kreitman RJ. Immunotoxins in cancer therapy. *Curr Opin Investig Drugs*. 2002;3:1089–1091.
- Kreitman RJ. Recombinant toxins for the treatment of cancer. *Curr Opin Mol Ther*. 2003;5:44–51.
- Crocker PR, Vark A. Siglecs, sialic acids and innate immunity. *Trends Immunol*. 2001;22:337–342.
- Hursey M, Newton DL, Hansen HJ, Ruby D, Goldenberg DM, Rybak SM. Specifically targeting the CD22 receptor of human B-cell lymphomas with RNA damaging agents: a new generation of therapeutics. *Leuk Lymphoma*. 2002;43:953–959.
- Nitschke L, Floyd H, Crocker PR. New functions for the sialic acid-binding adhesion molecule CD22, a member of the growing family of Siglecs. *Scand J Immunol*. 2001;53:227–234.
- Moyron-Quiroz JE, Partida-Sanchez S, Oñils-Hernández R, Sandoval-Monies C, Santos-Arquedo L. Expression and function of CD22, a B-cell restricted molecule. *Scand J Immunol*. 2002;55:343–351.
- Tadder TF, Tuscano J, Selo S, Kohn JH. CD22, a B lymphocyte-specific adhesion molecule that regulates antigen receptor signaling. *Annu Rev Immunol*. 1997;15:481–504.
- Hanna R, Ong GL, Mallett MJ. Processing of antibodies bound to B-cell lymphomas and other hematological malignancies. *Cancer Res*. 1996;56:3062–3068.
- Shan D, Press OW. Constitutive endocytosis and degradation of CD22 by human B cells. *J Immunol*. 1995;154:4466–4475.
- Adair JR, Athwal DS, Emtege JS. Humanized antibodies. *International Patent Publication* 1991; WO91/02967.
- Genentech. Rituxan label. Available at: <http://www.ntx.com/rituxan/pi.htm>. Accessed on June 1, 2003.

26. BIAevaluation version 3.0. Software Handbook. Uppsala, Sweden: BIAcore; 1997.
27. Dowell JA, Korh-Bradley J, Liu H, King SP, Berger MS. Pharmacokinetics of gemtuzumab ozogamicin, an antibody-targeted chemotherapy agent for the treatment of patients with acute myeloid leukemia in first relapse. *J Clin Pharmacol*. 2001;41:1206-1214.
28. Gibaldi M, Perrier D. *Pharmacokinetics*. 2nd ed. New York, NY: Marcel-Dekker; 1982.
29. French R, Powers U. Estimation of AUC and its variance in animal pharmacokinetic studies with partial concentration-time profiles. *Wyeth-Ayerst Biometrics Report 95031*. 1996;1-12.
30. Hinman LM, Hamann PR, Wallace R, Menéndez AT, Durr FE, Upešćić J. Preparation and characterization of monoclonal antibody conjugates of the calicheamicins: a novel and potent family of antitumor antibiotics. *Cancer Res*. 1993;53:3336-3342.
31. Danie NK, Frost P. Antibody-targeted chemotherapy with immunoconjugates of calicheamicin. *Curr Opin Pharmacol*. 2003;3:386-390.
32. 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;345:241-247.
33. Clynes RA, Towers TL, Presta LG, Ravetch JV. Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. *Nat Med*. 2000;6:443-446.
34. Manches O, Lui G, Chapout L, et al. In vitro mechanisms of action of rituximab on primary non-Hodgkin's lymphomas. *Blood*. 2003;101:949-954.
35. Leonard JP, Link BK. Immunotherapy of non-Hodgkin's lymphoma with hLL2 (aprafuzumab, an anti-CD22 monoclonal antibody) and Hu1D10 (apobuzumab). *Semin Oncol*. 2002;19:81-86.
36. Schindler J, Sausville E, Messmann R, Uhr JW, Vitetta ES. The toxicity of deglycosylated ricin A chain-containing immunotoxins in patients with non-Hodgkin's lymphoma is exacerbated by prior radiotherapy: a retrospective analysis of patients in five clinical trials. *Clin Cancer Res*. 2001;7:255-258.

ORIGINAL ARTICLE

John F. DiJoseph · Andrew Popplewell · Simon Tickle  
Heather Ladyman · Alastair Lawson · Arthur Kunz  
Kiran Khandke · Douglas C. Armellino  
Erwin R. Boghaert · Philip R. Hamann  
Karen Zinkewich-Peotti · Sue Stephens · Neil Weir  
Nitin K. Damle

## Antibody-targeted chemotherapy of B-cell lymphoma using calicheamicin conjugated to murine or humanized antibody against CD22

Received: 13 April 2004 / Accepted: 21 May 2004 / Published online: 30 July 2004  
© Springer-Verlag 2004

**Abstract** Antibody-targeted chemotherapy with immunoconjugates of calicheamicin is a clinically validated strategy in cancer therapy. This study describes the selection of a murine anti-CD22 mAb, m5/44, as a targeting agent, its conjugation to a derivative of calicheamicin (CalichDM) via either acid-labile or acid-stable linkers, the antitumor activity of CalichDM conjugated to m5/44, and its subsequent humanization by CDR grafting. Murine IgG1 mAb m5/44 was selected based on its subnanomolar affinity for CD22 and ability to be internalized into B cells. CalichDM conjugated to m5/44 caused potent growth inhibition of CD22<sup>+</sup> human B-cell lymphomas (BCLs) in vitro. The conjugate of m5/44 with an acid-labile linker was more potent than an acid-stable conjugate, a nonbinding conjugate with a similar acid-labile linker, or unconjugated CalichDMH in inhibiting BCL growth. CalichDM conjugated to m5/44 caused regression of established BCL xenografts in nude mice. In contrast, both unconjugated m5/44 and a nonbinding conjugate were ineffective against these xenografts. Based on the potent antitumor activity of m5/44-CalichDM conjugates, m5/44 was humanized by CDR grafting to create g5/44, an IgG4 anti-CD22

antibody. Both m5/44 and g5/44 bound CD22 with subnanomolar affinity. Competitive blocking with previously characterized murine anti-CD22 mAbs suggested that g5/44 recognizes epitope A located within the first N-terminal Ig-like domain of human CD22. Antitumor efficacy of CalichDM conjugated to g5/44 against BCL xenografts was more potent than its murine counterpart. Based on these results, a calicheamicin conjugate of g5/44, CMC-544, was selected for further development as a targeted chemotherapeutic agent for the treatment of B-cell malignancies.

**Keywords** B-lymphoid malignancies · CD22 · Cell surface molecules · Cytotoxic immunoconjugates · Humanized antibody · Targeted chemotherapy

### Abbreviations

|           |                                                           |
|-----------|-----------------------------------------------------------|
| AcBut     | 4-(4'-Acetylphenoxy) butanoic acid                        |
| AcPAc     | (3-Acetylphenyl) acetic acid                              |
| ATC       | Antibody-targeted chemotherapy                            |
| BCL       | B-cell lymphoma                                           |
| CalichDM  | N-Acetyl-γ-calicheamicin dimethyl disulfide derivative(s) |
| CalichDMA | CalichDM acid                                             |
| CalichDMH | CalichDM hydrazide                                        |
| CDR       | Complementarity determining region                        |
| NHL       | Non-Hodgkin's lymphoma                                    |
| PBMC      | Peripheral blood mononuclear cell                         |
| TAA       | Tumor-associated antigen                                  |

J. F. DiJoseph · K. Khandke · D. C. Armellino  
E. R. Boghaert · N. K. Damle (✉)  
Oncology Discovery, Wyeth Research,  
200/4604, 401 North Middletown Road,  
Pearl River, NY 10965, USA  
E-mail: damlen@wyeth.com  
Tel.: +1-845-6023984  
Fax: +1-845-6025557

A. Kunz · P. R. Hamann  
Chemical Sciences, Wyeth Research, Pearl River,  
New York, USA

A. Popplewell · S. Tickle · H. Ladyman · A. Lawson  
K. Zinkewich-Peotti · S. Stephens · N. Weir  
Celltech R&D, Slough, UK

### Introduction

Monoclonal antibodies (mAbs) targeted to tumor-associated antigens (TAAs) have been widely used in various experimental therapeutic approaches that include the

specific delivery of radioactive isotopes in the form of radioimmunotherapeutics, or of cytotoxic agents in the form of antibody-drug conjugates [1]. Antibodies or their fragments have been either chemically linked or genetically fused to xenotoxins to produce immunotoxins. In addition, anti-TAA mAb(s), chemically conjugated or genetically fused to enzymes, have been used to evaluate antibody-directed enzyme prodrug therapy [2]. Antibody-targeted chemotherapy (ATC) is now an accepted therapeutic strategy in which a cytotoxic agent is conjugated to an anti-TAA mAb that specifically binds to the TAA on the surface of tumor cells and is internalized, resulting in its intracellular delivery [3]. This strategy facilitates preferential delivery of the cytotoxic agent into the tumor cells and reduces nonspecific toxicity in normal tissues.

The ATC strategy was validated with the approval of gemtuzumab ozogamicin (Mylotarg or CMA-676) by the US FDA in May 2000 [4]. Gemtuzumab ozogamicin is a CD33-targeted immunoconjugate in which a humanized anti-CD33 mAb, hP67.6, is conjugated via an acid-labile AcBut hybrid linker to *N*-acetyl  $\gamma$ -calicheamicin dimethyl hydrazide (CalichDMH), a derivative of a potent cytotoxic agent,  $\gamma$ -calicheamicin [5-9]. Calicheamicin- $\gamma$  binds DNA in the minor groove and undergoes thiol-dependent structural changes in its enediyne moiety to generate a di-radical that abstracts hydrogens from the phosphodiester backbone of DNA causing double-strand DNA breaks leading to cell death [10-12]. Gemtuzumab ozogamicin is approved for the treatment of acute myeloid leukemia (AML) in elderly (≥60 years of age) patients in first relapse who are not candidates for other therapies [13].

The ATC strategy could be similarly applied to B-lymphoid malignancies using an immunoconjugate of *N*-acetyl  $\gamma$ -calicheamicin dimethyl disulfides (CalichDM) targeted to B-cell lineage-specific differentiation antigens. CD22 is a 130-kDa type 1 transmembrane glycoprotein that is selectively displayed on the surface of most mature B lymphocytes and their malignant counterparts [14]. It is not expressed on the surface of non-B lineages including hematopoietic stem cells and pre-B cells [15]. Similar to CD33, CD22 is a sialic acid-binding lectin (Siglec-2) and a member of the Ig superfamily [16, 17]. Its N-terminal IgV-like domain recognizes  $\alpha$ -2,6-linked terminal sialic acid residues on glycoproteins on the surface of cells that interact with B cells and regulates activation of B cells [15-17]. Antibodies bound to CD22 are rapidly internalized [18, 19] making CD22 ideally suited for targeted delivery of cytotoxic agents [20-22]. Using a murine anti-CD22 mAb, m5/44, as a CD22-targeting agent, the present study demonstrates potent antitumor activity of m5/44 conjugated to CalichDM. It further describes humanization of m5/44 by CDR grafting to generate g5/44, a humanized IgG4 anti-CD22 mAb used to create a CD22-targeted conjugate of CalichDMH. This conjugate of CalichDMH, like its murine counterpart, causes regression of established BCL xenografts in nude mice.

## Materials and methods

### Cell lines

B-cell lymphoma (BCL) lines Daudi (CCL-213), Raji (CCL-86), Ramos (CRL-1923), and RL (CRL-2261), and myeloid leukemia cell line HL-60 (CCL-240) were all obtained from the American Type Culture Collection (ATCC, Manassas, Va., USA). The cell lines were determined to be mycoplasma free as determined by a polymerase chain reaction mycoplasma detection assay (ATCC). The cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES, 1 mM sodium pyruvate, 0.2% glucose, penicillin G sodium 100 U/ml, and streptomycin sulfate 100 µg/ml.

### Mice

Female, BALB/c nu/nu (nude) mice (18-23 g) were obtained from Charles River Laboratories, Wilmington, Mass., USA and BALB/c mice from Harlan Laboratories, UK. All mice were provided with food and water ad libitum throughout the studies. All experimental procedures involving mice were approved by the Celltech Ethical Review Committee and Wyeth Animal Care and Use Committee according to established guidelines.

### Monoclonal antibodies and CD22 antigen

A number of purified murine anti-CD22 mAbs were obtained from their respective vendors: HD239, MYG13, RFB-4, SJ10.1H11 (Santa Cruz Biotech, Santa Cruz, Calif., USA); S-HCL1/anti-Leu 14 (Becton Dickinson, Franklin Lakes, N.J., USA), 4KB128 and To15 (Dako Corp, Carpinteria, Calif., USA). In addition, a panel of murine mAbs to human CD22 was developed using conventional hybridoma technology [23] and screened for isotype, cell binding, internalization on Daudi cells and on peripheral blood B cells, and binding affinity to human CD22-mFc antigen. A human CD22-mFc construct encoding the entire extracellular domain of human CD22 genetically fused to the Fc portion of murine IgG1 was prepared and expressed in NS0 cells. The humanized IgG4 anti-CD33 mAb, hP67.6, and its conjugate of CalichDMH, gemtuzumab ozogamicin (Mylotarg or CMA-676), have been described previously [5, 9].

### Cloning of m5/44 V-region genes

Total RNA was prepared from m5/44 hybridoma cells using the RNeasy kit (Qiagen, Valencia, Calif., USA). RNA was reverse transcribed to cDNA using the Clontech (Palo Alto, Calif., USA) cDNA Advantage

reverse transcriptase for polymerase chain reaction (PCR) kit. This cDNA was then used as the template for PCR using a set of "forward" degenerate primers designed to anneal to DNA encoding the conserved signal sequence, and a reverse primer annealing to DNA encoding the framework 4/constant region junction. PCR was performed using AmpliTaq enzyme and buffer (Applied Biosystems, Foster City, Calif., USA). The resultant PCR products were cloned into sequencing vectors (InVitrogen TOPO-TA cloning kit; InVitrogen, Carlsbad, Calif., USA), and the DNA sequence was determined. N-Terminal protein sequencing of the purified antibody (from hybridoma) was used to confirm that the translated sequences corresponded to the observed protein sequence (data not shown).

#### Plasmid construction and expression of chimeric constructs

The murine V-region sequences were cloned into the expression vectors pMR10.1 and pMR14 [24]. These are vectors designed for the expression of light and heavy chain, respectively, containing DNA encoding constant regions of human  $\kappa$  light chain and human  $\gamma$ -4 heavy chain. The VL DNA was subcloned into the light chain expression vector, creating plasmid pMR10 (544 cL). The VH DNA was subcloned into the heavy chain expression vector, creating plasmid pMR14 (544 cH). Transient cotransfection of both expression vectors into CHO-L761 cells generated chimeric c5/44 antibody. Transfections were carried out using Lipofectamine reagent according to manufacturer's instructions (InVitrogen, Carlsbad, Calif., USA). Mutations to CDR-H2 were carried out by a PCR approach, using oligonucleotides with specific mismatched bases to introduce desired codon changes.

#### Modulation of anti-CD22 mAb bound to B cells

Peripheral blood mononuclear cells (PBMCs; isolated from human blood) or BCL and anti-CD22 mAb were mixed ( $0.5 \mu\text{g}$  of mAb/ $5 \times 10^5$  cells/ml) and incubated on ice for 60 min after which cells were washed. The antibody-treated cell suspensions were then transferred to a  $37^\circ\text{C}$  water bath for timed intervals between 0 and 24 h. At the defined times, tubes with cells were immediately returned to iced ( $4^\circ\text{C}$ ) water. Tubes were centrifuged and the supernatant removed to quantify the presence of murine IgG by ELISA in order to confirm that the antibody bound to the cell surface was not shed in the surrounding medium. R-Phycoerythrin-conjugated donkey antimouse IgG (H+L chains) antibody (Jackson ImmunoResearch Laboratories, West Grove, Pa., USA) was added to the cell pellet, and samples were maintained in iced water for 1 h then washed as above. Cells were then resuspended in wash buffer and analyzed by flow cytometry to detect the antibody bound to the cells.

#### Conjugation of anti-CD22 mAb with calicheamicin

The murine anti-CD22 mAb m5/44 was covalently conjugated on the  $\epsilon$ -NH<sub>2</sub> groups of lysines to either CalichDMH with the acid-labile AcPAc ((3-acetylphenyl)acetic acid) linker or to N-acetyl  $\gamma$ -calicheamicin dimethyl acid (CalichDMA) with the acid-stable Amide linker [25]. The humanized version of m5/44, g5/44, was conjugated to CalichDMH with the acid-labile AcBut (4-(4'-acetylphenoxy) butanoic acid) linker [9]. Attempts to conjugate m5/44 with CalichDMH using the AcBut linker resulted in aggregated conjugates and thus were not evaluated. After the conjugation reaction, conjugated monomeric IgG antibody was separated from the aggregated conjugated antibody by gel permeation chromatography. Only the monomeric IgG conjugates as defined by the size-exclusion profile of the antibody conjugates were used in various investigations. The AcPAc-linked conjugates of m5/44 had an average calicheamicin loading of  $18 \mu\text{g}$  of CalichDMH per milligram of the antibody protein (1.6 M/M). The average drug loading of the Amide-linked calicheamicin conjugates was  $20 \mu\text{g}$  of CalichDMA per milligram of antibody (1.8 M/M). CMA-676 is comprised of humanized IgG4 anti-CD33 mAb, hP67.6, which is linked to CalichDMH via the AcBut linker with an average drug loading of  $35 \mu\text{g}$  of CalichDMH per milligram of hP67.6 protein (3.2 M/M). All conjugates were confirmed as having low endotoxin ( $< 5.0 \text{ EU/ml}$ ) as determined by the Limulus amoebocyte lysate test (BioWhittaker, Walkersville, Md., USA).

#### Cytotoxicity assays

Ramos and Raji BCL and HL-60 myeloid leukemic cell lines were cultured in the presence of various concentrations of unconjugated calicheamicin (CalichDMH), unconjugated anti-CD22 mAb m5/44, NAc- $\gamma$ -calicheamicin derivatives conjugated to m5/44, or CMA-676. Calicheamicin conjugate concentrations were based on the content of calicheamicin equivalents. Concentrations of unconjugated mAb were based on protein content. Ninety-six-hour cell viability was measured by propidium iodide (PI) exclusion and analyzed by flow cytometry (FACSort, Becton Dickinson Immunocytometry System; Becton Dickinson, San Jose, Calif., USA). The region for viable cells was set using both the forward light scatter and red fluorescence properties of the cells. Nonviable cells lose their membrane integrity, shrink in size, and allow diffusion of PI in the nucleus to bind cellular DNA resulting in lower forward light scatter and increased red fluorescence. The loss of viability was determined by the loss of cells from within the gated region defining viable cells [26]. The average number of viable cells per six replicate cultures was calculated. The percentage survival of BCL cells in these cultures was calculated using the following equation: percentage survival =  $100 \times (\text{number of viable cells in treated cul-})$

tures) / (number of viable cells in control cultures). IC<sub>50</sub>s (with 95% confidence intervals) were calculated by logistic nonlinear regression and are reported as the calicheamicin concentration from each treatment group that causes 50% loss of cell viability.

#### Humanization of m5/44 into g5/44

The methodology described by Adair et al. [27] was used to design CDR-grafted variants using selected human acceptor frameworks. Genes were designed to encode the grafted sequences gHa and gLa, and a series of overlapping oligonucleotides were designed and constructed. A PCR assembly technique was employed to construct the CDR-grafted V-region genes from these oligonucleotides [28]. The grafted variants gLb and gHb-d were constructed from the parental grafts gLa and gHa by PCR mutagenesis, using oligonucleotides with specific mismatched bases to introduce desired codon changes (see below).

#### Competition of CD22 binding among murine and humanized mAb

Murine antibody m5/44 (final concentration of 600 ng/ml) was added to humanized, grafted antibodies (in the range of 0.015–1.5 µg/ml) in a 96-well plate. Ramos cells ( $1.5 \times 10^5$ ) were added to the wells, and the plate was incubated on ice for 1 h. Control wells contained 1.5 µg/ml of either humanized grafted antibody or m5/44. The cells were washed twice with wash buffer (PBS + 5% FBS) after which secondary antibody, either RPE-conjugated donkey antimouse IgG (H + L chains) (Jackson ImmunoResearch Laboratories, West Grove, Pa., USA) or FITC-conjugated goat-antimouse IgG (H + L chains) (Zymed, South San Francisco, Calif., USA), was added and incubated on ice for 1 h. Cells were then washed with wash buffer and analyzed by flow cytometry.

#### Biosensor analysis

Biosensor analysis was carried out using a BIAcore 2000 (BIAcore AB, Uppsala, Sweden). CD22-mFc was covalently immobilized on the *N*-hydroxysuccinimide-activated carboxymethyl dextran-coated biosensor chip (CM5) using a standard amine-coupling chemistry at a protein density of approximately 2,000 resonance units. Samples of antibodies or their calicheamicin conjugates were diluted in the HBS buffer (10 mM HEPES, pH 7.4, containing 150 mM NaCl, 3 mM EDTA, and 0.005% polysorbate 20 [v/v]) and injected in the concentration range of 1–100 nM over the CD22-mFc-coated biosensor chip surface at a flow rate of 30 µl/min for 3 min to allow for binding. Dissociation of the bound antibody was monitored after washing the biosensor chip with the HBS buffer for 15 min. The antigenic surface was

regenerated by washing the biosensor chip with 15 µl of the regeneration buffer (10 mM NaOH and 200 mM NaCl) for 30 s, followed by a stabilization time of 2 min before the next cycle. Kinetic constants were calculated by nonlinear least-square regression analysis using a 1:1 Langmuir binding curve fitting model and BIAevaluation program (version 3.0; BIAcore).

#### Subcutaneous BCL xenografts

Female, athymic nude mice were exposed to total body irradiation (400 rad) to further suppress their residual immune system and facilitate the establishment of BCL xenografts. Three days later, irradiated mice were injected s.c. with  $1 \times 10^7$  Ramos or Raji cells suspended in Matrigel (Collaborative Biomedical Products, Bedford, Mass., USA) diluted 1:1 in RPMI 1640 medium, in the dorsal, right flank. When the tumors reached a mass of 0.3–0.6 g, they were staged to ensure uniformity of the tumor mass ( $n=7$  to 9 mice/group) prior to the administration of therapy. Conjugated CalichDM derivatives, unconjugated antibody (m5/44), or CMA-676 were administered i.p. in sterile saline (0.2 ml/mouse) on day 1 and the same treatment was repeated twice, 4 days apart (Q4D $\times$ 3). Doses of conjugated CalichDM were based on the quantity of calicheamicin equivalents. Length and width (in cm) of the tumors were measured at least once a week and their mass was calculated as tumor mass (g) =  $0.5 \times (\text{tumor width}^2) (\text{tumor length})$ . Mean ( $\pm$  SEM) tumor mass for each treatment group was calculated and compared to the vehicle-treated group for statistical significance using a one-sided *t*-test analysis of variance and subsequent pairwise comparisons to vehicle with the error term for the *t*-test based on the pooled variance across all treatment groups. Tumor mass values for each treatment group were recorded up to 50 days after the initiation of treatment or until either tumor-bearing mice died or the tumors grew to 15% of the body weight, at which time these mice were humanely euthanized according to institutional regulations. The number of tumor-free mice at the end of each study for each treatment group was also recorded.

## Results

#### Modulation of anti-CD22 mAb bound to B lymphoma cells

A panel of 17 murine mAbs was made against human CD22. Three antibodies from this panel (m5/44, m6/13, and m38) were selected for further evaluation based on their subnanomolar affinity for CD22-mFc ( $K_D=60$ –500 pM), and their ability to bind and modulate CD22 on the surface of Daudi BCL and PBMC cells (Fig. 1a, b). The modulation of surface-bound mAb as defined by the loss of surface display of the bound mAb was evaluated by flow cytometry.

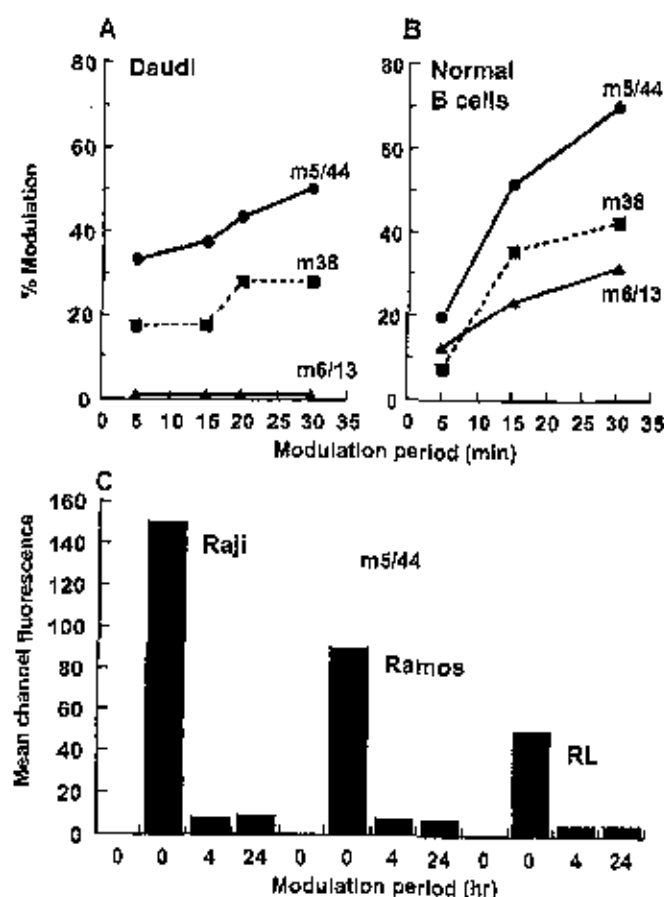


Fig. 1a-c Modulation of mAbs bound to CD22 expressed on the surface of normal peripheral blood-derived B cells and BCLs. Anti-CD22 mAbs were allowed to bind at 4°C to either Daudi BCLs (a) or normal peripheral blood B cells (b), and washed to remove unbound mAb. The B cells were further maintained at 37°C for varying lengths of time up to 30 min after which the surface retention of the anti-CD22 mAb was assessed by indirect immunofluorescence analysis. A similar evaluation was conducted using three distinct BCLs (c), except that the modulation period was extended to 24 h.

Shedding of mAbs from the cell surface as assessed by ELISA was shown to be below the limit of detection, consistent with their high-affinity binding to CD22. While each of the three mAbs bound similarly to CD22 on B cells (data not shown), mAb m5/44 demonstrated the maximum modulation, with 50% of the initial surface-bound m5/44 no longer detectable after 30 min. In contrast, mAb m6/13 bound to, but failed to modulate from, the Daudi BCL surface (Fig. 1a). Similar rank order of modulation (m5/44 > m38 > m6/13) was observed using PBMC (Fig. 1b). The modulation of the cell surface-bound m5/44 was further confirmed using CD22<sup>+</sup> Ramos, Raji, and RL BCLs (Fig. 1c). Each of these BCLs showed >90% modulation of the bound m5/44 over the 4-h period. Examination of these cells after 24 h failed to demonstrate any membrane reappearance of

m5/44. These observations suggest that m5/44, bound to the surface CD22, is rapidly and almost completely (>90%) modulated without recycling over the 24-h period. The lack of any detectable antibody in the culture supernatants, combined with the rapid modulation of the bound mAb, is indicative of the internalization of the mAb bound to the cell surface CD22. Based on its ability to bind human CD22 with high affinity ( $K_D = 200$  pM) and internalize (modulation without shedding) in BCLs, m5/44 was selected for further investigations.

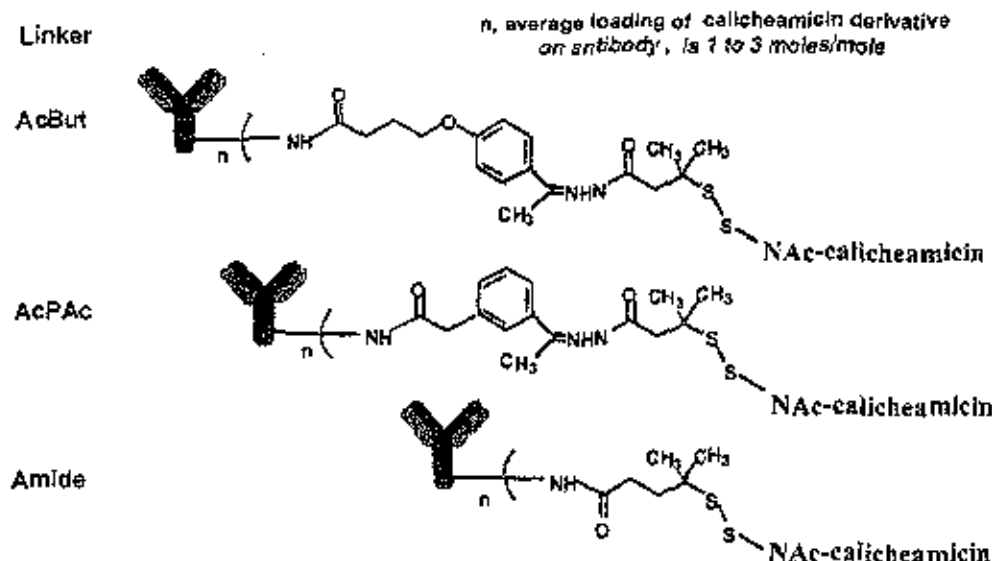
#### Calicheamicin conjugation of m5/44

Three linkers were used to covalently conjugate Calicheamicin derivatives to mAbs and the chemical structures of these are shown in Fig. 2. Calicheamicin can be conjugated to mAb using the AcBut or AcPac linker. The AcBut linker is the preferred linker based on our experience with the currently marketed agent, gemtuzumab ozogamicin (Mylotarg, CMA-676). Both the AcBut- and the AcPac-linked conjugates include an acid-labile hydrazone functional group that undergoes hydrolysis at similar rates under acidic conditions, releasing Calicheamicin from its covalently conjugated state [9]. Unlike the conjugation of humanized mAb to Calicheamicin via the AcBut linker, similar conjugation of murine mAb resulted in significant aggregation, rendering the murine mAb conjugates unusable. This problem was avoided by the use of the AcPac linker that allowed the generation of conjugates from which Calicheamicin can be released under acidic conditions. Thus, m5/44 was conjugated to Calicheamicin using the acid-labile AcPac linker and to Calicheamicin using the acid-stable Amide linker. CMA-676, an AcBut-linked conjugate of humanized anti-CD33 mAb, was used as a nonbinding negative control conjugate in experiments using CD22<sup>+</sup> CD33<sup>-</sup> BCLs.

#### In vitro cytotoxic activity of calicheamicin conjugated to anti-CD22 mAb

The effect of m5/44, conjugated to Calicheamicin with the AcPac and the Amide linkers, on the growth of CD22<sup>+</sup> CD33<sup>-</sup> Raji and Ramos BCLs was examined in vitro. Calicheamicin, unconjugated m5/44, and CMA-676 were used as controls. As shown in Table 1, both conjugates of m5/44 inhibited the growth of Ramos and Raji BCLs. The AcPac-linked conjugate of m5/44 was 4-fold to 8-fold more potent than the Amide-linked conjugate of the same mAb. The cytotoxic potency of the amide-linked conjugate of m5/44 and unconjugated Calicheamicin was similar. Unconjugated m5/44 had no effect on the growth of either BCL at concentrations up to 100 µg/ml, demonstrating that the potent growth-inhibitory effect of CD22-targeted conjugates was due to the cytotoxic activity of Calicheamicin. CD33-targeted CMA-676, in spite of the presence of the acid-labile

**Fig. 2** Structural representation of the linkers used to conjugate CalichDM derivatives to the targeting mAb. Both the AcBut- and AcPac-linked conjugates include an acid-labile hydrazone functionality. The amide-linked conjugate lacks such an acid-labile group



AcBut linker, was 20- to 260-fold less potent in inhibiting BCL growth than the CD22-targeted AcPac-linked conjugate of m5/44. In contrast, CMA-676 was 166-fold more potent than the CD22-targeted conjugate with acid-labile linker in inhibiting the growth of CD22<sup>+</sup>CD33<sup>+</sup> HL-60 myeloid leukemic cells. These results demonstrate that CD22-targeted delivery of CalichDM can produce a potent and specific cytotoxic activity against CD22<sup>+</sup> BCLs.

#### Antitumor efficacy of CD22-targeted calicheamicin immunoconjugate against B-lymphoma xenografts

CalichDM conjugates of m5/44 were examined for their antitumor efficacy against s.c. Ramos or Raji BCL xenografts established in nude mice. Conjugates of CalichDM derivatives were administered i.p. as three doses injected 4 days apart (Q4D $\times$ 3) [25] in BCL xenograft-bearing mice. The antitumor activity of CalichDM conjugates against Ramos BCL xenografts and Raji BCL xenografts are shown in Figs. 3 and 4, respectively. CalichDM conjugated to m5/44 via either the AcPac or

the Amide linker at a dose of 160  $\mu$ g/kg of conjugated CalichDM inhibited BCL growth in both models. This dose of conjugated CalichDM has been shown to be lower than its maximum nonlethal dose and optimal for its antitumor activity [25]. In contrast, the nonbinding conjugate, CMA-676, and m5/44 (administered at a dose of 8 mg/kg equivalent to the antibody quantity in its calicheamicin conjugates) were ineffective against the BCL xenografts. After confirming the ability of m5/44 to target and deliver CalichDM to BCLs and cause potent anti-BCL activity, m5/44 was humanized to create g5/44 for further evaluation as a CD22-targeted drug-delivery agent.

#### Cloning and expression of chimeric 5/44 antibodies

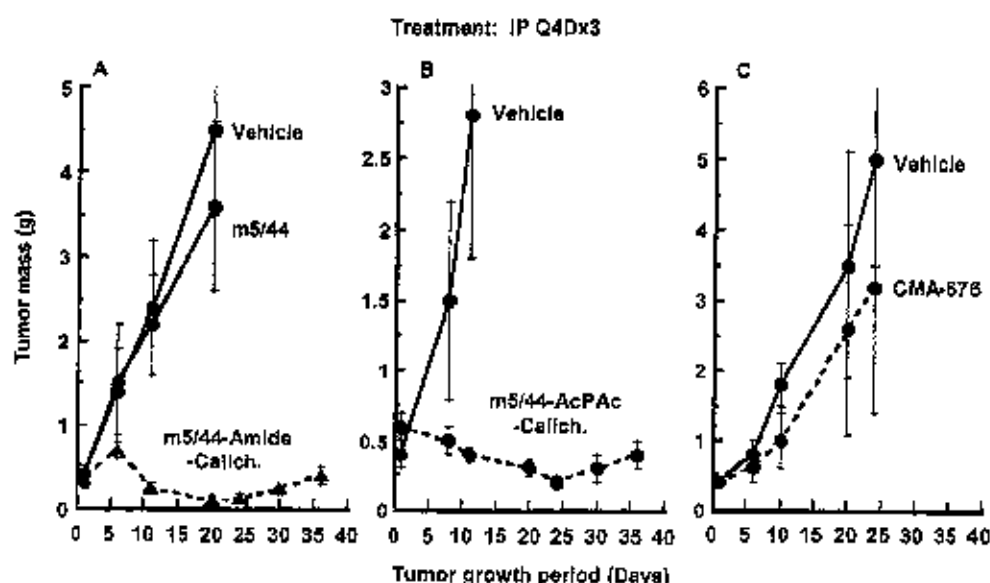
A PCR approach was used to clone the V-region genes from hybridoma m5/44, and the DNA sequence was determined and translated to reveal the protein sequence (Fig. 5). Vectors expressing a chimeric version of this antibody, with the mouse V-regions and human constant regions ( $\kappa$  light chain,  $\gamma$ -4 heavy chain) were

**Table 1** Cytotoxic activity of calicheamicin conjugates against human BCLs in vitro. Human B lymphoma cells were cultured for 96 h in the presence of various concentrations of CalichDM derivatives conjugated to m5/44 with either the Amide linker or the AcPac linker, CD33-targeted CMA-676, or CalichDMH, after

which the viable cell number in each culture was enumerated by their exclusion of PI and detected by flow cytometry. Concentrations of the conjugates of CalichDM derivatives were based on the calicheamicin equivalents (nM) and the unconjugated mAb m5/44 was used based on its protein concentration

| Treatment                                  | IC <sub>50</sub> (nM of calicheamicin equivalents) |                                           |                                          |
|--------------------------------------------|----------------------------------------------------|-------------------------------------------|------------------------------------------|
|                                            | CD22 <sup>+</sup> CD33 <sup>+</sup> Raji           | CD22 <sup>+</sup> CD33 <sup>+</sup> Ramos | CD22 <sup>+</sup> CD33 <sup>+</sup> HL60 |
| Anti-CD22 mAb m5/44 unconjugated           | > 100 $\mu$ g/ml                                   | > 100 $\mu$ g/ml                          | Not determined                           |
| CalichDMH                                  | 0.41                                               | 0.055                                     | 0.5                                      |
| Anti-CD22 m5/44-amide-CalichDMA            | 0.34                                               | 0.041                                     | > 67.6                                   |
| Anti-CD22 m5/44-AcPac-CalichDMH            | 0.08                                               | 0.005                                     | 7.5                                      |
| Anti-CD33 hP67.6-AcBut-CalichDMH (CMA-676) | 1.63                                               | 1.285                                     | 0.045                                    |

**Fig. 3A-C** Effect of CalichDM conjugated to m5/44 on the growth of Ramos BCL xenografts. Subcutaneous tumors were established prior to the administration of CalichDM conjugates at a fixed dose of 160  $\mu$ g of conjugated CalichDM/kg. Three such doses were administered 4 days apart (Q4Dx3). CD33-targeted conjugate of CalichDMH (CMA-676) was used as a nonspecific conjugate. Unconjugated m5/44 at 8 mg/kg was used as a control. Three separate studies are presented in panels A, B, and C. Results are presented as mean tumor mass  $\pm$  SEM



assembled. The m5/44 VH sequence (Fig. 5a) revealed a potential N-linked glycosylation site in CDR-H2 (residue N55). In addition, a lysine residue within CDR-H2 (K60), a potential site for calicheamicin conjugation, was of concern, since calicheamicin conjugation close to the antigen-binding site might be expected to interfere with its binding to CD22. The following constructs were made to assess the effect of removal of these residues: cH-N55Q, cH-T57A, and cH-K60R. The N55Q and T57A changes ablate the glycosylation site by changing the amino acid sequence away from the consensus N-X-T/S; the K60R mutation replaces the potentially reactive

lysine with the similarly positively charged residue arginine. The resultant cH variant plasmids were co-transfected with the cL plasmid to generate expressed chimeric antibody variants. They were then analyzed for binding affinity to CD22-mFc protein using a BIAcore assay (Table 2). Removal of the glycosylation site in construct T57A resulted in a 2- to 3-fold faster on-rate and a significantly slower off-rate compared with the chimeric, giving an overall improvement in affinity of approximately 3- to 5-fold. The N55Q mutation had no effect on affinity. This result was unexpected since removal of any carbohydrate alone (the N55Q change) apparently had no effect on its binding to CD22, therefore the improved affinity requires the specific T57A change. One possible explanation is that regardless of the presence of carbohydrate, the threonine at position 57 exerts a negative effect on binding that is removed on conversion to alanine. Replacement of lysine at position 60 with arginine (K60R) had no effect on affinity of the antibody, and this change removed a potential reactive site that might have negatively impacted the affinity of the antibody after its conjugation with calicheamicin. Both these mutations (T57A and K60R) were therefore retained in the CDR-H2 sequence of the humanized heavy chains.

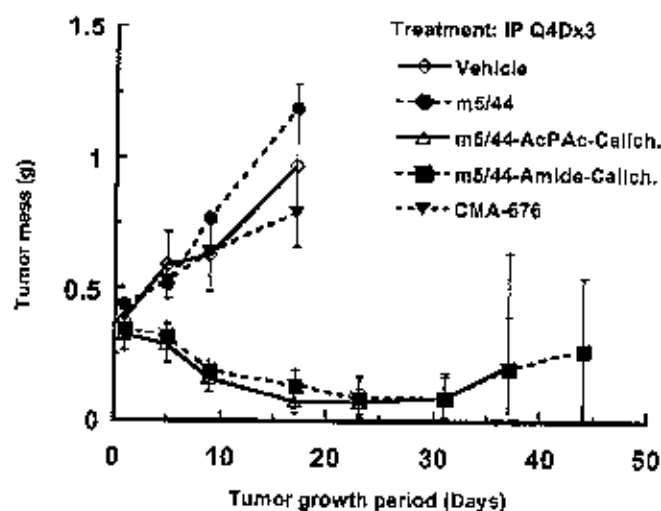


**Fig. 4** Effect of CalichDM conjugated to m5/44 on the growth of Raji BCL xenografts. Subcutaneous tumors were established to an average mass of 300 mg after which CalichDM conjugates of m5/44 were administered at 160  $\mu$ g of conjugated CalichDM/kg. Three such doses were administered 4 days apart (Q4Dx3). The CD33-targeted conjugate of CalichDMH (CMA-676) was used as a nonspecific conjugate. Unconjugated m5/44 at 8 mg/kg was used as a control. Results are presented as mean tumor mass  $\pm$  SEM

#### Humanization strategy

Germline acceptor frameworks were selected from human VH subgroup I (VH1-3/DP-7) [29] and human VK subgroup I (O12/DPK9) [30]. For the heavy chain, the framework 4 acceptor sequence was derived from human J-region sequence JH-4; the human JK-1 sequence was used for the light chain framework 4. Figure 5 shows the protein sequences of the m5/44 heavy and light chains compared with the human acceptor frameworks, and also the designed grafted sequences. The

Fig. 5a,b The sequences of the m5/44 variable heavy region (VH, VL) are aligned with the human germline sequence (DP-7, DPK-9). Asterisks indicate differences between mouse (donor) and human (acceptor) residues. CDRs are indicated in blue (not shown for acceptor frameworks), mouse framework residues are colored red, and human residues are shown in black. The grafted gH and gL sequences are shown below the human germline sequence. Sequences underlined and colored red indicate donor residues that have been retained in the graft. The underlined residues in CDR-H2 show potential sites for glycosylation and calicheamicin conjugation. Residue numbering is according to Kabat [31]. The variable domain sequences of the light and heavy chains of the murine antibody m5/44 and its humanized counterparts have been deposited in the Genbank with the following accession number: m5/44 kappa light chain VJ region, AY531629 (protein ID number AAS45201), g5/44 kappa light chain VJ region gLa, AY531630 (protein ID number AAS45202), g5/44 kappa light chain VJ region gLb, AY531631 (protein ID number AAS45203), m5/44 heavy chain VDJ region, AY531632 (protein ID AAS45204), g5/44 heavy chain VDJ region gHa, AY531633 (protein ID number AAS45205), g5/44 heavy chain VDJ region gHb, AY531634 (protein ID number AAS45206), g5/44 heavy chain VDJ region gHc, AY531635 (protein ID number AAS45207), g5/44 heavy chain VDJ region gHd, AY531636 (protein ID number AAS45208)

### A) 5/44 Heavy Chain Sequence Alignment

|       |                           |                                  |                |      |     |
|-------|---------------------------|----------------------------------|----------------|------|-----|
|       | 10                        | 20                               | 30             | 40   | 50  |
| VH    | EVQLQQSGTFLARPGASVKMSCKAS | GYRFTNYWIE                       | WVKQRPGQGLEWIG | GINP |     |
| DP-7  | QVQLVQSGAEVKKPGASVKVCKAS  |                                  | WVRQAPGQGLEWMG |      |     |
| gHa-d | EVQLVQSGAEVKKPGASVKVCKAS  | GYRFTNYWIE                       | WVRQAPGQGLEWIG | GINP |     |
|       | 60                        | 70                               | 80             | 90   | 100 |
| VH    | GNNYTTYRRNLKG             | KATLTAVTSASTAYMDLSSLSSEDSAVYYCTR | EGYGNVY        |      |     |
| DP-7  | KPQG                      | RVMTADTSTSTVYMESSLRSEDTAVYYCAR   |                |      |     |
| gHa   | GNNYATYRRNLKG             | RATLTADTSTSTVYMESSLRSEDTAVYYCTR  | EGYGNVY        |      |     |
| gHb   | GNNYATYRRNLKG             | RVMTADTSTSTVYMESSLRSEDTAVYYCTR   | EGYGNVY        |      |     |
| gHc   | GNNYATYRRKPGQ             | RATLTADTSTSTVYMESSLRSEDTAVYYCTR  | EGYGNVY        |      |     |
| gHd   | GNNYATYRRKPGQ             | RVMTADTSTSTVYMESSLRSEDTAVYYCTR   | EGYGNVY        |      |     |
|       | 110                       |                                  |                |      |     |
| VH    | AWFAY                     | WGQGTLLVTVSS                     |                |      |     |
| JH4   |                           | WGQGTLLVTVSS                     |                |      |     |
| gHa-d | AWFAY                     | WGQGTLLVTVSS                     |                |      |     |

### B) Light Chain Sequence Alignment

|       |                         |                                  |                 |
|-------|-------------------------|----------------------------------|-----------------|
|       | 10                      | 20                               | 40              |
| VL    | DVVVTQTPLSLFVSGDQVSLSC  | RSSQSLANSYGNITFLS                | WYLHKPGQSPQLLIY |
| DPK9  | DIQMTQSPSSLSASVGDRVTITC |                                  | WYQKPKGRAPKLLIY |
| gLa   | DVQYVTSFSSLSASVGDRVTITC | RSSQSLANSYGNITFLS                | WYLHKPGKAPQLLIY |
| gLb   | DVVVTQSPSSLSASVGDRVTITC | RSSQSLANSYGNITFLS                | WYLHKPGKAPQLLIY |
|       | 60                      | 70                               | 80              |
| VL    | GISNRFS                 | GVPDRFSGSGSGTDFTLTISKIKPEDLGMYIC | LOGTHQPYT       |
| DPK9  |                         | GVPDRFSGSGSGTDFTLTISLQPEDFATYYC  |                 |
| gLa-b | GISNRFS                 | GVPDRFSGSGSGTDFTLTISLQPEDFATYYC  | LOGTHQPYT       |
|       | 100                     |                                  |                 |
| VL    | FGGGTKLEIKR             |                                  |                 |
| JK1   | FGGGTKVEIKR             |                                  |                 |
| gLa-b | FGGGTKVEIKR             |                                  |                 |

CDRs are shown according to a combination of the sequence hypervariability definition [31] and the structural definition [32]. In practice, this means that the larger sequence-based limits apply to all CDRs except CDR-H1, which comprises residues H26-H35. The alignment shows that there are 22 framework differences between the donor and acceptor heavy chain frameworks. The light chain alignment shows that there are 27 framework differences between the donor and acceptor sequences. At each position of sequence difference, an analysis was made of the potential of the murine residue to contribute to antigen binding, either directly or indirectly through effects on packing or at the VH/VL

Table 2 Binding of chimeric 5/44 and its mutants to CD22. Human CD22-mFc fusion protein was captured on antimouse IgG Fc-specific antibody-coated biosensor chip. The binding of chimeric 5/44 and its three mutants to captured CD22-mFc was studied using BIAcore. The above mutations were localized in the CDR2 of the variable domain of the heavy chain of chimeric 5/44

| Mutation in CDRH2 | $K_a$ ( $\times 10^5$ ), 1/Ms | $K_d$ ( $\times 10^{-4}$ ), 1/s | $K_D$ ( $\times 10^{-10}$ ), M | $\sim K_D$ , nM |
|-------------------|-------------------------------|---------------------------------|--------------------------------|-----------------|
| Murine VH         | 2.9                           | 1.1                             | 3.9                            | 0.4             |
| N55Q              | 5.8                           | 1.9                             | 3.3                            | 0.3             |
| T57A              | 7.8                           | 0.5                             | 0.7                            | 0.07            |
| K60R              | 5.0                           | 1.0                             | 2.0                            | 0.2             |

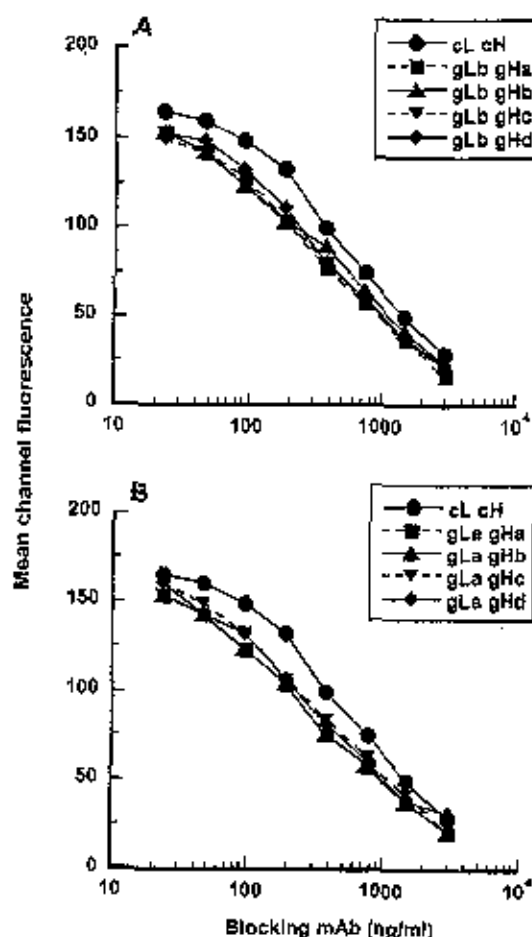


Fig. 6a,b Competitive inhibition of the binding of murine anti-CD22 mAb, m5/44, to Ramos BCL by its humanized versions assembled by combinations of four distinct grafts of heavy chain (gHa-d) and two distinct grafts of light chain (gLa, gLb). Increasing concentrations of the humanized mAb were mixed with the fixed concentration of m5/44 and then allowed to bind to Ramos BCL. Binding of the murine mAb, m5/44, was monitored by indirect immunofluorescence analysis using flow cytometry. The chimeric version of m5/44 (cL cH) was also used in this evaluation as a positive control.

interface. If a murine residue was considered important and sufficiently different from the human in terms of size, polarity or charge, then that murine residue was back-mutated into the human acceptor sequence. In this way, four VH grafts and two VK grafts were designed, as shown in the Fig. 5. Grafts gHc and gHd contain three additional human residues at the C-terminal end of CDR-H2. This portion of the CDR is not at the antigen-binding surface, and so introduction of human residues can be tolerated.

#### Characterization of humanized variants of anti-CD22 mAb

The vectors encoding grafted variants were cotransfected into CHO cells in a variety of combinations, and the

expressed humanized mAbs were compared with the chimeric 5/44 antibody. Binding activity was assessed in a competition assay, competing with the binding of the original m5/44 antibody to Ramos BCL (Fig. 6). The binding of each of the eight grafted variants to cell surface CD22 was indistinguishable from that of the others. This suggests that heavy chain residues A67 and L69, and light chain residue V3 are not important for antigen binding and can be replaced by the human residues. Each graft was found to perform more effectively than the chimeric antibody at competing against m5/44. Hence CD22-binding activity was not diminished by the CDR-grafting process, and was slightly improved. This modest improvement is most likely due to the T57A change present in each heavy chain graft. The introduction of the three additional human residues at the end of CDR-H2 (gHc and gHd) did not affect CD22 binding of the grafted mAb.

Since all the grafted variants were fully active, the graft combination of gLa-gHd with the least number of murine residues was selected for further studies. The light chain graft gLa has six donor framework residues. Residues V2, V4, L37, and Q45 are potentially important packing residues. Residue H38 is at the VH/VL interface. Residue D60 is a surface residue close to the CDR-L2 and may directly contribute to the antigen binding. The heavy chain graft gHd has four donor framework residues. Residues E1 and A71 are surface residues close to the CDRs. Residue I48 is a potential packing residue. Residue T93 is present at the VH/VL interface. Of these residues, E1 and A71 are found in other germline genes of human subgroup I. The selected humanized IgG4 mAb with V-regions gLa and gHd is referred to as g5/44.

#### Identification of CD22 epitope recognized by g5/44

The ability of humanized anti-CD22 mAb, g5/44, to inhibit the binding of various murine anti-CD22 mAbs to CD22 was evaluated by biosensor analysis using a BIAcore (Fig. 7). Many of these murine mAbs have been characterized with respect to their binding to defined epitopes on the extracellular domain of human CD22 [33-36]. As expected, g5/44 binding to CD22-mFc blocked subsequent binding of m5/44. In addition, binding of two other anti-CD22 mAbs—HD239 and S-HCL1/anti-Leu14 (recognizing epitope A located in the first Ig-like domain of CD22) [34, 35]—was also inhibited by g5/44 bound to CD22-mFc. In contrast, three other anti-CD22 mAbs—RFB4 (recognizing epitope B located on the third Ig-like domain of CD22), 4KB128 (recognizing the second Ig-like domain of CD22), and T015 (recognizing epitope C on the fourth Ig-like domain of CD22) [34, 35]—were still able to bind to CD22-mFc in the presence of g5/44. Prior binding of g5/44 to CD22-mFc also inhibited the subsequent binding of anti-CD22 mAbs, SJ10.1H11, MYG13, and m6/13. These results suggest that g5/44 and its murine counterpart, m5/44, both rec-

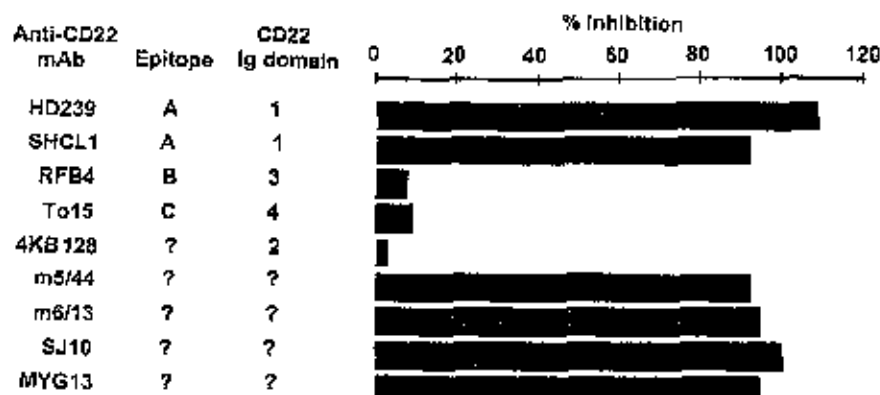


Fig. 7 Effect of humanized anti-CD22 mAb g5/44 on the binding of various murine anti-CD22 mAbs to human CD22-mFc detected by biosensor analysis. Various murine anti-CD22 mAbs were individually (50  $\mu$ g/ml) allowed to bind CD22-mFc immobilized on biosensor chip that had been pretreated (100  $\mu$ g/ml) with either anti-CD22 mAb g5/44 or anti-CD33 mAb hP67.6 (control mAb). Percentage inhibition of murine mAb binding was calculated as  $100 \times [1 - (\text{RU with g5/44 pretreatment}) / (\text{RU with hP67.6 pretreatment})]$

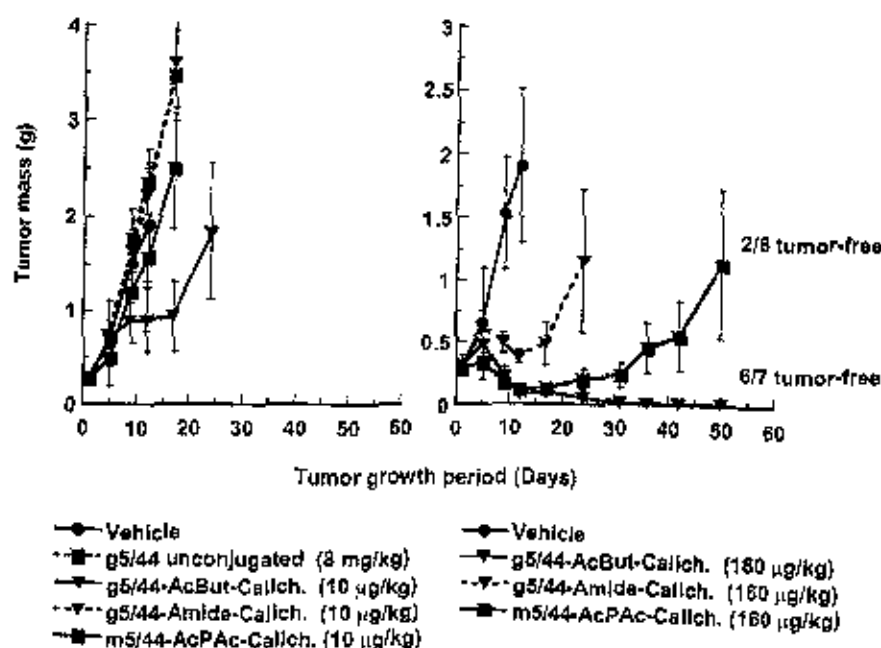
recognize epitope A located in the first N-terminal domain of CD22. The CD22 domain(s) reactive with anti-CD22 mAbs MYG13 and SJ10.1H11 was unknown. However, their blocking by g5/44 suggests that these mAbs also recognize the first Ig-like domain of CD22.

Antitumor activity of calicheamicin conjugated to humanized anti-CD22 mAb g5/44

Humanized anti-CD22 mAb g5/44 was conjugated to CalichDM using either the Amide or the AcBut linker.

Incubation of Ramos BCLs in vitro with CalichDM conjugated to g5/44 via either acid-labile or acid-stable linkers inhibited their growth in vitro with potency similar to that observed with its murine counterparts (data not shown). The ability of CalichDM derivatives conjugated to either m5/44 or g5/44 to inhibit the growth of Ramos BCL xenografts was examined using both the acid-labile (AcPac for m5/44 and AcBut for g5/44) and the acid-stable (Amide) linker-bearing conjugates. When administered i.p., Q4Dx3 at a dose of 160  $\mu$ g of conjugated CalichDM/kg, each of the above conjugates caused significant inhibition ( $p < 0.05$ ) of the growth of Ramos BCL xenografts (Fig. 8). At this dose, CalichDMH conjugated to g5/44 via the AcBut linker caused greater and longer lasting inhibition of Ramos BCL growth (85% of mice being tumor-free after 50 days) than the conjugate of m5/44 prepared with the AcPac linker (28% of tumor-bearing mice being tumor-free after 50 days) ( $p < 0.05$  on day 50). In experiments not shown here, the AcBut-linked conjugate of g5/44 often caused long-term regression of established BCL

Fig. 8 Effect of calicheamicin conjugates of murine and humanized anti-CD22 mAbs. Subcutaneous BCLs were established prior to the i.p. administration of CalichDM conjugates at either 10 or 160  $\mu$ g of conjugated CalichDM/kg. Three such doses were administered 4 days apart (Q4Dx3). Unconjugated humanized anti-CD22 mAb, g5/44, was administered i.p. at 8 mg/kg Q4Dx3 as a control. Results from a representative experiment are presented as mean tumor mass (g)  $\pm$  SEM. The number of tumor-free mice is indicated wherever appropriate



xenografts without any evidence of re-growth of the tumors for up to 125 days. Among mice treated with a lower dose of conjugated CalichDM (10  $\mu$ g/kg Q4Dx3), only mice treated with the AcBut-linked conjugate of g5/44 demonstrated significantly smaller tumor masses compared with those in the vehicle-treated mice ( $p < 0.05$ ). The degree of inhibition of BCL growth with the acid-labile AcBut conjugate of g5/44 was greater than that with its acid-stable Amide conjugate ( $p < 0.05$  on day 25). The treatment with unconjugated g5/44 (8 mg/kg) had no effect on the growth of Ramos BCL. Together, these results provide convincing evidence of antitumor activity of CD22-targeted conjugates of CalichDM derivatives against human BCL xenografts.

## Discussion

Tumor-targeted delivery of calicheamicin, as a conjugate of a mAb reactive against an internalizable TAA, is now a clinically validated therapeutic strategy exemplified by gemtuzumab ozogamicin [5-7]. CD33, the target of gemtuzumab ozogamicin, is a myeloid lineage-specific antigen and is expressed on the vast majority of myeloid malignancies. In order to apply similar antibody-targeted calicheamicin therapy to B-lymphoid malignancies, we focused on the CD22 molecule. CD22 is expressed on the surface of mature B lymphocytes and their malignant counterparts but not their precursors and is known to be internalized upon binding to anti-CD22 mAb [18, 19]. Based on its efficient internalization, CD22 has been the focus of intense investigations for the targeted delivery of immunotoxins and radioimmunotherapy [20-22, 37].

Consistent with the ability of CD22 to facilitate targeted delivery of cytotoxic agents, we have recently described the preclinical antitumor activity of a CD22-targeted immunoconjugate of calicheamicin, CMC-544 [38]. This immunoconjugate comprises a humanized anti-CD22 antibody, g5/44, conjugated to CalichDMH via the AcBut linker [38]. The present study serves as a prelude to the above study describing CMC-544 and represents the evolution of various studies beginning with the selection of murine antibody against human CD22, its ability to modulate cell-surface CD22, its conjugation with calicheamicin, and evidence of the antitumor activity of its calicheamicin conjugates. In addition, the present study also described the humanization of the murine anti-CD22 mAb, epitope mapping of the humanized antibody, g5/44, and, lastly, the evidence of antitumor efficacy of calicheamicin-conjugated humanized anti-CD22 antibody.

To apply a CD22-targeted chemotherapy strategy, a panel of murine anti-human CD22 mAbs were made and evaluated for their affinity of binding to human CD22 on B cells and their ability to cause its internalization. Based on its high-affinity binding to CD22 and also its ability to modulate CD22, mAb m5/44 (murine IgG1) was selected for further studies. CalichDM was conjugated to m5/44

using either the acid-labile AcPac or acid-stable Amide linker as shown in Fig. 2. The presence of acid-labile hydrazide functionality in the AcBut- or AcPac-linked conjugates allows, under acidic conditions (pH range of 4.5-6) such as those in the lysosomes, rapid release of CalichDMH from its conjugated state. Binding of the immunoconjugate to the readily internalizable target antigen makes possible efficient intracellular presentation of the conjugated cytotoxic drug.

Some cells may internalize the immunoconjugate by antigen-nonspecific pinocytosis even in the absence of the target antigen. In addition, the immunoconjugates with acid-labile linkage of the drug may release the drug, even in the absence of their internalization, upon acidification of the extracellular environment of metabolically active growing cells. The antibody conjugates with acid-labile linkers that do not bind to the target cells may, therefore, exert some cytotoxic activity in an antigen-nonspecific manner. In contrast, the Amide-linked conjugates may require more extensive "intracellular processing" of the immunoconjugate in order to release the drug. Therefore, in order to be effective, the Amide-linked conjugates of calicheamicin are required to be internalized via specific or nonspecific mechanisms by the target cells. This may explain why the cytotoxic activity of the amide-linked conjugates is often lower than that of AcBut- or AcPac-linked conjugates as shown in this study.

Both acid-labile and acid-stable conjugates of m5/44 were effective against CD22<sup>+</sup> BCLs. The AcPac-conjugated m5/44 was 4- to 8-fold more potent than the Amide-conjugated m5/44 whose cytotoxic activity was similar to that of unconjugated CalichDMH. Irrespective of the linker used, the CD22-targeted conjugates of calicheamicin were 5- to 30-fold more potent than the nontargeted (antigen-nonspecific) conjugate, CMA-676 bearing the acid-labile AcBut linker. The potent cytotoxic activity of the Amide-linked conjugate of m5/44 is consistent with its efficient internalization by CD22<sup>+</sup> BCLs. These results further indicate that the intracellular delivery of CalichDMH resulting from the CD22-mediated internalization of its antibody conjugate followed by the hydrolytic release of CalichDMH is more efficient in inhibiting BCL growth than nontargeted mechanisms of delivery of CalichDMH such as passive diffusion.

Calicheamicin conjugates of m5/44 were also evaluated for their antitumor activity against human Raji and Ramos BCL xenografts in nude mice. Both AcPac-linked and Amide-linked conjugates of m5/44 were equally effective in inhibiting the tumor growth. In contrast, both unconjugated m5/44 and the CD33-targeted conjugate, CMA-676 (used as a nonbinding control), had no effect on the growth of BCL xenografts. The unconjugated m5/44 was administered at a dose of 8 mg/kg equivalent to the antibody quantity in its calicheamicin conjugates. The antitumor activity of the m5/44 calichDMH conjugates is, therefore, not due to the antibody component of the conjugate. Doses higher than 8 mg/kg

of the unconjugated m5/44 were not administered, so it is not absolutely clear that the naked antibody is completely devoid of antitumor activity. However, the anti-CD22 mAb m5/44 is a murine IgG1 isotype mAb, and the murine IgG1 isotype is unable to fix complement and binds poorly to Fc receptors. Thus, m5/44 would not be expected to mediate ADCC or CDC.

Unconjugated CalichDMH at nonlethal doses had been consistently ineffective in the human tumor xenograft models evaluated so far [39] (E. Boghaert, J. Di-Joseph, and C. Discafani, unpublished observations). In the absence of its tumor-targeting, the poor antitumor activity of CalichDMH is not surprising, and further underscores the therapeutic advantage conferred by the tumor-targeted delivery of cytotoxic agents such as calicheamicin. m5/44 was also evaluated for binding to cryopreserved B-NHL tumor biopsy samples. All of the B-cell NHL samples tested ( $n = 50$ ), including high-grade diffuse, mantle cell and follicular NHL, reacted positively with m5/44 (J. Crocker, Birmingham Heartlands Hospital, Birmingham, UK, unpublished observations). This demonstration of m5/44 binding to B-NHL biopsy samples supports its use as a vehicle for targeted delivery of cytotoxic agents such as calicheamicin to CD22<sup>+</sup> tumor cells. Based on its binding to B-NHL biopsy samples and potent anti-BCL activity of its conjugate of calicheamicin, m5/44 was selected for preclinical development.

To reduce or eliminate its immunogenicity in humans, the murine m5/44 antibody was humanized by CDR grafting [40]. Variable region heavy and light chain sequences of murine mAb m5/44 exhibited highest homology to human subgroup I of V<sub>H</sub> and subgroups I and II of V<sub>K</sub>. Hence, the germline frameworks of human V<sub>H</sub> subgroup I (DP7) and human V<sub>K</sub> subgroup I (DPK9) were selected, and murine CDRs were grafted on the human frameworks. For both heavy and light chain, framework 4 acceptor sequences were derived from human J-region sequences JH-4 and JK-1, respectively. Six distinct grafts were expressed and shown to be equivalent to the original anti-CD22 mAb m5/44 in terms of affinity and competition for binding to CD22 on B cells. In addition, a potential N-linked glycosylation site and a lysine residue in the V<sub>H</sub> CDR2 (a potential target for calicheamicin conjugation) were removed to avoid potential interference during binding to CD22.

One such graft, gLb-gHd, was selected to assemble a full antibody with the human IgG4 constant region. This humanized anti-CD22 mAb was referred to as g5/44. The heavy chain amino acid sequences of g5/44 are 91% identical to that of hP67.6, the humanized IgG4 anti-CD33-targeting agent in gemtuzumab ozogamicin. Even if the targeting mAb is humanized, the presence of drug-linker in the mAb conjugate may induce Ab response in patients against the CalichDMH AcBut linker portion of the molecule. However, it is reassuring that no antibodies targeted to either CalichDMH, AcBut linker, or the entire immunoconjugate were detected in 142 patients who received gemtuzumab ozogamicin in a phase

II study [5]. The choice of the human IgG4 constant region in g5/44 was also based on its poor effector capabilities (ADCC and CDC) [41, 42], and hence, the therapeutic effect observed with its conjugate of calicheamicin can be attributed entirely to the targeted delivery to, and subsequent cytotoxicity of, calicheamicin against tumor cells.

Various murine anti-CD22 mAbs have been used to define distinct epitopes located on the Ig-like domains within the extracellular region of human CD22 [34, 35]. Blocking studies using various murine mAbs revealed that humanized anti-CD22 mAb g5/44 inhibits the binding of only those murine anti-CD22 mAbs that had previously been mapped to bind to the first N-terminal Ig-like (IgV) domain of human CD22 [34, 35]. This suggests that g5/44 and its murine counterpart, m5/44, both recognize epitope A located in the N-terminal IgV domain of CD22. Epratuzumab, a humanized IgG1 anti-CD22 mAb (hLL2), is presently being evaluated as a naked antibody therapeutic [43] and also as a CD22-targeted radioimmunotherapeutic agent for the treatment of B-NHL [37]. This mAb has been shown to bind epitope B on human CD22, originally identified by the binding of a murine anti-CD22 mAb, RFB4 [36]. Thus, g5/44 and epratuzumab appear to recognize distinct epitopes on CD22.

Our primary objective with g5/44 is to use it as a CD22-targeted calicheamicin-delivery agent in the treatment of B-lymphoid malignancies, and we have shown here a potent antitumor activity of CalichDMH conjugated to g5/44 against BCL xenografts. We have no evidence to support other potential mechanisms of action, as g5/44 has a human IgG4 isotype and does not demonstrate any ADCC or CDC activity in vitro using a human source of complement and peripheral blood mononuclear cells as effector cells (L. Kalyandrug, unpublished observation) or antitumor effect in vivo ([38] and this study). Some anti-CD22 antibodies have, however, been shown to mediate apoptosis and growth inhibition of BCLs. The N-terminal IgV domain of CD22 is known to bind the natural ligand of CD22,  $\alpha$ -2,6-linked sialic acid termini on glycosylated cell-surface structures. Some but not all mAbs reactive with the N-terminal IgV of CD22 have been shown to block the ligand binding by CD22 [34]. One such mAb, HD22-7, caused apoptosis and inhibited the growth of BCL xenografts in nude mice [44]. Whether g5/44 (or m5/44) can inhibit ligand binding by CD22 has not been determined. However, if the ability of an anti-CD22 mAb to cause BCL apoptosis [44] is closely tied with its ability to block ligand binding by CD22 [34], then g5/44 is not expected to be a blocking antibody capable of neutralizing the CD22/ligand interaction.

Our antibody-targeted chemotherapy strategy derives its advantages from the cytotoxic potency of calichDM, high-affinity binding of the targeting antibody conjugate to the targeted TAA, and the antibody-conferred long half-life of the conjugated drug in circulation. Antitumor activity of the mAb-CalichDMH conjugate is

independent of the effector functional capabilities of the targeting antibody. The demonstration of the potent antitumor activity of the calicheamicin conjugates of g5/44 supports its evaluation as a CD22-targeted calicheamicin therapeutic agent in the treatment of B-NHL and other B-lymphoid malignancies. Based on its superior antitumor efficacy, an AcBut-linked conjugate of g5/44 and CalichDMH, designated as CMC-544 [38], has been selected for further development. CMC-544, also known as inotuzumab ozogamicin, is currently being evaluated in phase I clinical trials in B-NHL.

**Acknowledgements** We would like to thank Dr John Crocker of Birmingham Heartlands Hospital, Birmingham, UK, for the evaluation of m3/44 binding to the NHL biopsies; Dr Lyka Kalyandrug for studies with effector functions of antibodies; Fred Immerman for statistical evaluation of results; and Maureen Dougher and Latha Sridharan for assistance with various studies.

## References

- Trail P, Bianchi A (1999) Monoclonal antibody drug conjugates in the treatment of cancer. *Curr Opin Immunol* 11:584
- Dubowchik G, Walker M (1999) Receptor-mediated and enzyme-dependent targeting of cytotoxic anti-cancer drugs. *Pharmacol Ther* 83:67
- Damle NK, Frost P (2003) Antibody-targeted chemotherapy with immunocjugates of calicheamicin. *Curr Opin Pharmacol* 3:386
- Brass PF, Beltz J, Chen G, Chen XH, Duffy E, Kieffer L, Roy S, Sridhara R, Rahman A, Williams G, Pazdur R (2001) Gemtuzumab ozogamicin: approval summary: gemtuzumab ozogamicin in relapsed acute myeloid leukemia. *Clin Cancer Res* 7:1490
- Sievers E, Larson R, Stadtman E, Estey E, Lowenberg BH, Dombret HC, Karanes C, Theohald M, Bennett JM, Sherman ML, Berger MA, Eten CB, Loken MR, van Dongen JJM, Bernstein ID, Appelbaum FR (2001) Efficacy and safety of gemtuzumab ozogamicin in patients with CD33-positive acute myeloid leukemia in first relapse. *J Clin Oncol* 19:3244
- Larson R, Boogaerts M, Estey E, Karanes C, Stadtman EA, Sievers EL, Mineur P, Bennett JM, Berger MS, Eten CB, Muntean M, Loken MR, van Dongen JJM, Bernstein ID, Appelbaum FR (2002) Antibody-targeted chemotherapy of older patients with acute myeloid leukemia in first relapse using Mylotarg (gemtuzumab ozogamicin). *Leukemia* 16:1627
- Berger M, Leopold L, Dowell J, Korth-Bradley J, Sherman M (2002) Licensure of gemtuzumab ozogamicin for the treatment of selected patients 60 years of age or older with acute myeloid leukemia in first relapse. *Invest New Drugs* 20:395
- Hamann P, Hinman L, Beyer C, Lindh D, Upeslacijs J, Flowers DA, Bernstein I (2002) An anti-CD33 antibody-calicheamicin conjugate for treatment of acute myeloid leukemia: choice of linker. *Bioconjug Chem* 13:40
- Hamann P, Hinman L, Hollander L, Beyer CF, Lindh D, Holcomb R, Hallett W, Tsou HR, Upeslacijs J, Schohal D, Mountain A, Flowers DA, Bernstein I (2002) Gemtuzumab ozogamicin, a potent and selective anti-CD33 antibody-calicheamicin conjugate for treatment of acute myeloid leukemia. *Bioconjug Chem* 13:47
- Zein N, Sinha A, McGahren W, Ellestad G (1988) Calicheamicin 7B: an antitumor antibiotic that cleaves double-stranded DNA site specifically. *Science* 240:1198
- Lee MD, Dunne TS, Chang CC, Siegel MM, Morton GO, Ellestad GA, McGahren WJ, Borders DB (1992) Calicheamicins, a novel family of antibiotics. 4: Structural elucidations of calicheamicins. *J Am Chem Soc* 114:385
- Thorson J, Sievers E, Ahlert J, Shepard E, Whitman RE, Onwueme KC, Ruppen M (2000) Understanding and exploiting nature's chemical arsenal: the past, present and future of calicheamicin research. *Curr Pharm Des* 6:1841
- Mylotarg label. <http://www.fda.gov/cder/foi/label/2000/211741bl.pdf>
- Moyron-Quiroz JE, Partida-Sanchez S, Donis-Hernandez R, Sandoval-Montes C, Santos-Argumedo L (2002) Expression and function of CD22, a B-cell restricted molecule. *Scand J Immunol* 55:343
- Tedder TF, Tuscano J, Sato S, Kehrl JH (1997) CD22, a B lymphocyte-specific adhesion molecule that regulates antigen receptor signaling. *Ann Rev Immunol* 15:481
- Crocker PR, Varki A (2001) Siglecs, sialic acids and innate immunity. *Trends Immunol* 22:337
- Nitschke L, Floyd H, Crocker PR (2001) New functions for the sialic acid-binding adhesion molecule CD22, a member of the growing family of Siglecs. *Scand J Immunol* 53:227
- Hanna R, Ong GL, Mattes MJ (1996) Processing of antibodies bound to B-cell lymphomas and other hematological malignancies. *Cancer Res* 56:3062
- Shan D, Press OW (1995) Constitutive endocytosis and degradation of CD22 by human B cells. *J Immunol* 154:4466
- Kreitman RJ, Wilson WH, Bergeron K, Raggio M, Stettler-Stevenson M, Fitzgerald DJ, Pastan I (2001) Efficacy of the anti-CD22 recombinant immunotoxin BL22 in chemotherapy-resistant hairy-cell leukemia. *N Engl J Med* 345:241
- Pastan I, Kreitman RJ (2002) Immunotoxins in cancer therapy. *Curr Opin Investig Drugs* 3:1089
- Hursey M, Newton DL, Hansen HJ, Ruby D, Goldenberg DM, Rybak SM (2002) Specifically targeting the CD22 receptor of human B-cell lymphomas with RNA damaging agents: a new generation of therapeutics. *Leuk Lymphoma* 43:953
- Galfre G, Howe SC, Milstein C (1977) Antibodies to major histocompatibility antigens produced by hybrid cell lines. *Nature* 266:550
- Morgan A, Jones ND, Nesbitt AM, Chaplin L, Bodmer MW, Emtage JS (1995) The N-terminal end of the CH2 domain of chimeric human IgG1 anti-HLA-DR is necessary for C1q, Fc gamma RI and Fc gamma RIII binding. *Immunology* 86:319
- Hinman LM, Hamann PR, Wallace R, Menendez AT, Dur FE, Upeslacijs J (1993) Preparation and characterization of monoclonal antibody conjugates of the calicheamicins: a novel and potent family of antitumor antibiotics. *Cancer Res* 53:3336
- Coligan JE, Cruisbeck AM, Margulies DH, Shevach EM, Strober W (eds) (1992) Current protocols in immunology, vol 2. Wiley, New York
- Adair JR, Athwal DS, Emtage JS (1991) Humanised antibodies. International Patent Publication WO/91/09967
- Owens RJ, Young RJ (1994) The genetic engineering of monoclonal antibodies. *J Immunol Methods* 168:149
- Tomlinson IM, Walter G, Marks JD, Llewellyn MB, Winter G (1992) The repertoire of human germline VH segments reveals about fifty groups of VH segments with different hypervariable loops. *J Mol Biol* 227:776
- Cox JPL, Tomlinson IM, Winter G (1994) A directory of human germ-line V kappa segments reveals a strong bias in their usage. *Eur J Immunol* 24:327
- Kabat EA, Wu TT, Perry HM, Gottesman KS, Foeller C (1991) Sequences of proteins of immunological interest, 5th edn. Public Health Service, National Institutes of Health, Bethesda, MD
- Chothia C, Lesk AM, Tramontano A, Levitt M, Smith-Gill J, Air G, Sheriff S, Padlan EA, Davies D, Tulip WR, Colman PM, Spinelli S, Alzari PM, Poljak RJ (1989) Conformations of immunological hypervariable regions. *Nature* 342:877
- Li JL, Shen GL, Ghetie MA, May RD, Till M, Ghetie V, Uhr JW, Janossy G, Thorpe PE, Amlot P (1989) The epitope specificity and tissue reactivity of four murine monoclonal anti-CD22 antibodies. *Cell Immunol* 118:85

34. Engel P, Wagner N, Miller AS, Tedder TF (1995) Identification of the ligand-binding domains of CD22, a member of the immunoglobulin superfamily that uniquely binds a sialic acid-dependent ligand. *J Exp Med* 181:1581
35. Engel P, Wagner N, Smith H, Tedder TF (1995) Structure/function analysis of CD22: domains that mediate adhesion. In: *Leukocyte typing V*. Oxford University Press, Oxford, pp 526-527
36. Stein R, Belisle E, Hansen HJ, Goldenberg DM (1993) Epitope specificity of the anti-(B cell lymphoma) monoclonal antibody, LL2. *Cancer Immunol Immunother* 37:293
37. Griffiths GL, Govindan SV, Sharkey RM, Fisher DR, Goldenberg DM (2003)  $^{90}\text{Y}$ -DOTA-hLL2: an agent for radioimmunotherapy of non-Hodgkin's lymphoma. *J Nucl Med* 44:77
38. DiJoseph JF, Armellino DC, Boghaert E, Khandke K, Dougher Sridharan L, Kunz A, Hamann PR, Gorovits B, Udata C, Moran JK, Popplewell AG, Stephens S, Frost P, Darnie NK (2004) Antibody-targeted chemotherapy with CMC-544: a CD22-targeted immunoconjugate of calicheamicin for the treatment of B lymphoid malignancies. *Blood* 103:1807
39. Hinman LM, Hamann PR, Upešlacijs J (1995) Preparation of conjugates to monoclonal antibodies. In: *Borders DB, Doyle TW (eds) Endiync antibiotics as antitumor agents*. Dekker, New York, pp 87-106
40. Co MS, Queen C (1991) Humanized antibodies for therapy. *Nature* 351:501
41. Burton DR, Woof JM (1992) Human antibody effector function. *Adv Immunol* 51:1
42. Greenwood J, Clark M, Waldmann H (1993) Structural motifs involved in human IgG antibody effector functions. *Eur J Immunol* 23:1098
43. Leonard JP, Coleman M, Ketas JC, Chadburn A, Ely S, Funnian RR, Wegner WA, Hansen HJ, Ziccardi H, Eschenberg M, Gayko U, Cessano A, Goldenberg DM (2003) Phase I/II trial of epratuzumab (humanized anti-CD22 antibody) in indolent non-Hodgkin's lymphoma. *J Clin Oncol* 21:3051
44. Tuscano JM, O'Donnell RT, Miers LA, Kroger LA, Kukis DL, Lamborn KA, Tedder TF, DeNardo GL (2003) Anti-CD22 ligand-blocking antibody HB22.7 has independent lymphomacidal properties and augments the efficacy of  $^{90}\text{Y}$ -DOTA-peptide-Lym-1 in lymphoma xenografts. *Blood* 101:3641

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : B00176089-2

RECIBO OFICIAL DE CAJA : 08 - 11,218  
FECHA : FEBRERO 11 DE 2008

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 04-120655-00011-0000

LA PRESENTE CERTIFICACION  
HA SIDO EMITIDA POR EL  
S/S FASENACIONALI  
FOLIOS 39

## \*\*\*\*\* CONSIGNACION \*\*\*\*\*

| DEPOSITANTE       | TIPO PAGO    | BANCO           | CUENTA    | No. PAGO | FECHA PAGO | Vr. PAGO       |
|-------------------|--------------|-----------------|-----------|----------|------------|----------------|
| CAVELIER ABOGADOS | CONSIGNACION | BANCO DE BOGOTA | 062754387 | 2678193  | 11/02/2008 | \$1,814,500.00 |

## \*\*\*\*\* CONCEPTO \*\*\*\*\*

| CONCEPTO                                     | TOTAL CONCEPTO |
|----------------------------------------------|----------------|
| 1 50005-01-03 CAMBIO DE MODALIDAD Y MODIFICA | 103,000.00     |
| 12 MARCAS Y LEMAS COMERCIALES                | 103,000.00     |
| TOTAL :                                      | \$ 103,000.00  |

SON: CIENTO TRES MIL PESOS

RESPONSABLE :

RECIBO DE CAJA APLICADO AL EXPEDIENTE No. \_\_\_\_\_

Cdo 13115-841003-8

285  
~~288~~

**SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO**  
**DIVISIÓN DE NUEVAS CREACIONES**

Abogado  
**EMILIO FERRERO**

|        |            |           |
|--------|------------|-----------|
| ASUNTO | Radicación | 04-120665 |
|        | Trámite    | 002       |
|        | Evento     | 001       |
|        | Actuación  | 430       |
|        | Oficio     |           |
|        | Folios     | 3 0666    |

|                         |                          |                               |                                                           |
|-------------------------|--------------------------|-------------------------------|-----------------------------------------------------------|
| Patente de Invención    | <input type="checkbox"/> | Patente de Modelo de Utilidad | <input type="checkbox"/>                                  |
| Vía Nacional            | <input type="checkbox"/> |                               |                                                           |
| Vía PCT (fase nacional) | <input type="checkbox"/> | Cap. I                        | <input type="checkbox"/> Cap. II <input type="checkbox"/> |

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 le comunica:

**1. Documentos estudiados**

- 1.1. Descripción: Folios: 1 a 132 como se radicó inicialmente
- 1.2. Reivindicaciones: 1 a 45, Folios 133 a 139 como se radicó inicialmente  
1 a 46, Folios 156 a 163 modificadas  
1 a 16, Folios 175 a 178 modificadas  
1 a 8, Folio 255 modificadas
- 1.3. Prioridad: GB 0210121 de Mayo 2 de 2002

Se acepto divisional de esta solicitud y se le asignó el número de radicación 04-120665A. Por tanto, la solicitud 04-120665, sobre la cual se emite este concepto técnico, corresponde a la solicitud madre.

**2. Objeto de la invención:**

Titulo de la invención: "Anticuerpos específicos para CD22 humano y sus usos terapéuticos y diagnósticos"

El objeto de la presente solicitud se refiere a un anticuerpo específico para CD22 que comprende un dominio variable de cadena ligera 5/44-gL1 (SEQ ID N° 19) y la región variable de cadena pesada 5/44-gH7 (SEQ ID N° 27). Esta molécula tiene una cadena ligera que comprende la secuencia dada en la SEQ ID N°28 y la cadena pesada que consiste de la secuencia de la SEQ ID N°30.

También se reclaman los vectores y las células huésped que comprende la secuencia de la molécula antes descrita, así como la composición farmacéutica que la contiene.