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DIRECCIÓN DE NUEVAS CREACIONES

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MARGARITA CASTELLANOS
info@castellanosyco.com

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Patente de Invención Vía Nacional	☐	Patente de Modelo de Utilidad	☐		
Vía PCT (fase nacional)	☐	Cap.I	☐	Cap. II	☐

No. Solicitud Internacional PCT/JP2007/072099
Fecha de Presentación Internacional 14 Noviembre 2007

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

1. Documentos estudiados

Descripción:	Folios 69 a 138	27/Mayo/2009
Reivindicaciones:	Folios 139 a 140	27/Mayo/2009
Dibujos:	Folios 156 a 167	27/Mayo/2009
Secuencias:	Folios 142 a 155	27/Mayo/2009
Prioridad:	JP 2006-308482	14/Noviembre/2006

2. Análisis de la Prioridad

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

3. Objeto de la invención

Título de la solicitud: "Aptamero contra midcina y uso del mismo"

El problema planteado en esta solicitud consiste en suministrar moléculas capaces de inhibir la actividad de midcina

Para resolver este problema técnico la solicitud en estudio proporciona aptameros que antagonizan con micina, un complejo que comprende un aptamero que se une e

inhibe la actividad de midcina y una sustancia funcional como una sustancia de afinidad, de marcaje, una enzima, una droga que desarrolle un medio, un inhibidor de migración o un agente de diagnóstico.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K38/00; A61P35/00; A61P43/00; A61P9/00; C12N15/09; C12N15/115; G01N33/53; G01N33/566

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

El objeto de la reivindicaciones 13 y 15 a no es patentable de acuerdo con el Art citado, ya que corresponden a métodos de tratamiento

5. De la Solicitud de Patente

Título

El título incluye la expresión "uso" la cual no es patentable bajo el Art. 14 D486, por lo cual se sugiere al Solicitante hacer la corrección necesaria.

Reivindicaciones (Art 30 D 486, Art 22 PCT)

- La reivindicación 1 no es clara por que no define la estructura del aptámero reclamado, de la misma manera las reivindicaciones 2 a 7 no especifican la estructura del aptámero de interés y pretenden caracterizarlo por un resultado a alcanzar, se recuerda al solicitante que debe especificar las secuencia y demás características esenciales de la molécula que desea proteger.
- La reivindicación 8 no es clara por que: el literal b. no especifica cuales son los nucleótidos "sustituidos, deletados, insertado, añadidos", el solicitante debe especificar cuales son las modificaciones exactas que cumplen con las características esenciales del a invención. Por otra parte la palabra "deletados" no existe en español, es solicitante debe sustituirla por suprimidos. Por otra parte tanto el literal a como el b hacen referencia a que los nucleótidos contenidos en el áptamero son tales que las bases 2' ya sean pirimidinas o purinas "son átomos de flúor o sustituidos por átomos o grupos seleccionados del grupo que consiste de átomos de hidrogeno, grupos hidroxi y grupos metoxi". No es claro para el examinador como un nucleótido es remplazado por un átomo de flúor o por alguno de los grupos seleccionados, si el solicitante se refiere a que dichos nucleótidos tienen sustituciones con los grupos mencionados debe modificar la redacción de la reivindicación para que esta sea precisa, y ya que las sustituciones son las mismas ya sea pirimidina o purina eliminar los numerales i y ii. Por último, en la descripción solo se encuentra sustentada la sustitución con flúor (folio 100 línea 17 a 20), no se encuentra mención de sustituciones con los otros grupos mencionados, además se divulga que las SEQ ID NO: 3 (tabla 2) y SEQ ID NOs: 21, 34, 37, 38, 65 (tablas 3 y 4) no presentan la actividad deseada. Se sugiere al solicitante restringa el alcance de la reivindicación a lo divulgado en la descripción.
- Las reivindicaiones 9 a 12 no son claras por que se refieren a complejos y fármacos pero no proporcionan las características esenciales de dichas composiciones. Se recuerda al solicitante que estas composiciones deben ser

caracterizadas por todos sus componentes y sus respectivas proporciones, además la reivindicación 11, no tiene sustento en al descripción, donde no se divulga el efecto de los complejos mencionado ni se especifica la estructura de "la sustancia de afinidad" utilizada.

- El método de la reivindicación 16 no es claro por que no especifica los pasos secuenciales, ni lo reactivos ni materiales necesarios para llevar a cabo la detección del aptámero de interés, el solicitante debe proporcionar información suficiente para que la invención se reproducible.

6. Determinación del Estado de la Técnica

El Estado de la Técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: 14/Noviembre/2006, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación*
D1	Cancer Research 61, 8486 – 8491	Antisense Oligodeoxynucleotide Targeted to Midkine, a Heparin-binding Growth Factor, Suppresses Tumorigenicity of Mouse Rectal Carcinoma Cells	1/Diciembre/2001
D2	Nature Biotechnology Volume 22 Number 6, p649	Oligo oligarchy—the surprisingly small world of aptamers	Junio/2004

D1
Divulga reactivos antitumorales que inhiben la actividad de midcina.
http://cancerres.aacrjournals.org/content/61/23/8486.full.pdf+html

D2 divulga aptameros que consisten en oligonucleótidos los cuales son químicamente idénticos a las drogas antisentido.
http://www.ncbi.nlm.nih.gov/pubmed/15175673

7. Examen de Patentabilidad (Art 14 D486)

Nivel Inventivo (Art18 D486)

La reivindicación 1 consiste en "Un aptamero que posee una actividad inhibidora contra midcina "

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1	D5
Aptámero		Apatámeros para el tratamiento de cancer
Actividad inhibidora de midcina	Oligonucleótidos con actividad inhibiora de midcina	

Los documentos D1 y D2 se consideran el estado de la técnica cercano a la

invención definida en la reivindicación 1 porque:

D1 divulga oligonucleotidos antisentido fosforotioato-modificados cuyo objetivo es la midcina en sus fragmentos N- o C-terminal (ver figura 1), dichos oligonucleotidos fueron administrados a células CMT-93 que no expresaron pleiotrophin, Dichos oligonucleótidos presentaron efectos inhibitorios *in vivo*, el estado de la técnica demostró que los oligonucleótidos antisentido administrados con colágeno A suprimen el crecimiento de células tumorales *in vivo* y que por lo tanto, los oligonucleótidos antisentido dirigidos a midcina representan las drogas farmacéuticas,

D2 divulga aptámeros que son oligonucleótidos los cuales son químicamente idénticos a las drogas antisentido con aplicaciones terapéuticas (ver página 1).

La diferencia entre la invención, definida en la reivindicación 1 y D1 es que la invensción se refiere específicamente a aptámeros, mientras D1 divulga oligonucleótidos que inhiben la actividad de midcina pero no hace referencia espcifica a aptameros.

La diferencia entre la invención, definida en la reivindicación 1 y D2 es que la invención se refiere específicamente a aptámeros que inhiben midcina, mientras D2 divulga en forma general los aptámeros, su estructura y usos terapéuticos.

El efecto que logra el incluir la utilización de aptámeros que inhiben la actividad de midcina es su mayor especificidad, disminuyendo los efectos secundarios en el tratamiento de enfermedades relacionadas con la actividad de midcina.

Por lo tanto, el Problema Técnico Objetivo que pretende resolver esta invención se puede formular así: "cómo modificar el los oligonucleotidos divulgados en D1 para como aptámeros para que tengan mayor afinidad a su objetivo (la midcina) y no a otras proteínas presentes".

Sin embargo, la utilización de aptameros para aumentar la especifidad de oligonucleotidos en el tratamiento de enfermedades como el cáncer fue previamente divulgado en D2 (tabla 1 y en general el texto).

En consecuencia, la persona del oficio normalmente versada en la materia, incluiría los aptameros divulgados en D2 en el procedimiento de D1 para llegar así al objeto de la reivindicación 1 de la solicitud. Por lo cual se considera obvia.

El mismo razonamiento puede aplicarse para las reivindicaciones dependientes 2 a 7 y 9 a 15, que tienen las mismas características esenciales, y aquellas que no se encuentran divulgadas en D1 o D2 no tienen sustento en la descripción, tal como se menciono anteriormente.

8. Conclusión

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

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	Cancer Research 61, 8486 – 8491	1 a 7 y 9 a 15	Nivel Inventivo
D2	Nature Biotechnology Volume 22 Number 6, p649	1 a 7 y 9 a 15	Nivel Inventivo

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

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

JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha 28 NOV. 2012

Elaborado por: Sandra Carolina Crespo Cárdenas
Revisado Por: Consuelo Leguizamon Leguizamon
Aprobado por: José Luis Salazar López

Antisense Oligodeoxynucleotide Targeted to Midkine, a Heparin-binding Growth Factor, Suppresses Tumorigenicity of Mouse Rectal Carcinoma Cells

Yoshifumi Takei, Kenji Kadomatsu, Seiichi Matsuo, et al.

Cancer Res 2001;61:8486-8491.

[CANCER RESEARCH 61, 8486–8491, December 1, 2001]

Antisense Oligodeoxynucleotide Targeted to Midkine, a Heparin-binding Growth Factor, Suppresses Tumorigenicity of Mouse Rectal Carcinoma Cells[1]

Yoshifumi Takei, Kenji Kadomatsu, Seiichi Matsuo, Hiroshi Itoh, Kunihiko Nakazawa, Shunichiro Kubota, and Takashi Muramatsu[2]

Departments of Biochemistry [Y. T., K. K., T. M.] and Internal Medicine [S. M.], Nagoya University School of Medicine, Nagoya 466-8550, Koken Bioscience Institute, Tokyo 164-0072 [H. I.], and Department of Physiological Chemistry and Metabolism, Graduate School of Medicine, University of Tokyo, Tokyo 113-0033 [K. N., S. K.], Japan

ABSTRACT

Midkine (MK), a heparin-binding growth factor, is overexpressed in a wide range of human carcinomas and is believed to contribute to tumorigenesis and tumor progression. To develop an antitumor reagent, we designed a phosphorothioate antisense oligodeoxynucleotide molecule based on the secondary structure of MK mRNA. The antisense MK at the dosage of 5 μM suppressed MK production by CMT-93 mouse rectal carcinoma cells after cationic liposome-mediated transfection, to 13% of that in control cultures. The growth of CMT-93 cells and their colony formation in soft agar were inhibited by the addition of the antisense MK, whereas the control reagent, the sense MK, showed no effects. On s.c. injection into nude mice, CMT-93 cells transfected with the antisense MK formed tumors much smaller than those by control cells. Finally, untreated CMT-93 cells were inoculated to nude mice, and 7 days later the antisense MK (50 μM) with atelocollagen was directly injected into the preformed tumor region to evaluate the curative effect; the injection was repeated at the interval of 2 weeks. During the period of 10–41 days after initiation of therapy, the rate of increase of tumor volume treated with the antisense MK was found to be about 4.2-fold lower than that seen after treatment with the sense MK. On this occasion, proliferation of tumor cells as estimated by 5-bromodeoxyuridine incorporation was strongly inhibited, whereas angiogenesis was less affected. These findings strongly suggested the usefulness of MK antisense oligodeoxynucleotide as a new reagent for cancer therapy.

INTRODUCTION

Overexpression of growth factors and components of their signaling systems is observed frequently in carcinomas and serves as the principal cause of carcinogenesis in many cases (1). Thus, growth factors and their receptors have become one of the major targets to develop new modes of therapy for carcinoma. Antisense ODNs,[3] ribozymes, RNA, or ribozymes targeting these molecules have yielded beneficial results to suppress tumor growth both *in vitro* and *in vivo*. Typical examples are vascular endothelial growth factor, basic fibroblast growth factor and its receptor in melanoma (2, 3), and insulin-like growth factor-I receptor, hepatocyte growth factor, and tumor necrosis factor in glioblastoma (4–6). However, growth factor-targeted therapy of malignancy is still at the initial stage. The range of malignancies that can be treated is restricted, and injection of reagents after tumor formation has been effective in only a few cases (3, 7).

MK, a heparin-binding growth factor (8, 9), appears to be an excellent target of cancer therapy. MK is a M_r 13,000 protein rich in basic amino acids and cysteine (8, 9), and shares ~50% sequence identity with another heparin-binding growth factor, PTN (10), also called heparin-binding growth associated molecule (11). MK is overexpressed in a variety of tumors such as esophageal, gastric, colon, pancreatic, hepatocellular, lung, breast, and urinary bladder carcinomas (12–17), neuroblastoma (15) and Wilms' tumor (12), whereas in normal adult tissue, its expression is usually low or undetectable (10, 12). On transfection of the cDNA, MK transforms NIH3T3 cells (18). MK promotes survival (19, 20), growth (21, 22), and migration (23–25) of many cells. These activities in combination are believed to contribute to oncogenesis and tumor progression. Indeed, the affinity-purified antibody to MK partly suppressed growth of Wilms' tumor cells in culture (22). The present study was performed to develop a more potent antitumor reagent. For this purpose, we designed MK antisense ODNs and examined their anticancer activity against a rectal carcinoma cell line with particular attention to treatment of established tumors by intratumoral injection. For *in vivo* treatment, we adopted a new gene transfer method using a biomaterial, A. collagen, prepared from bovine dermal collagen (26), because antisense effects with the aid of cationic lipids in culture are not always reproducible *in vivo* (27).

MATERIALS AND METHODS

ODNs. Phosphorothioate-modified ODNs were synthesized with an automated solid-phase nucleotide synthesizer (Expdite8900 Nucleic Acid Synthesis System; Applied Biosystems) and subsequently purified using a Wakopak Handy ODS column (Waters). MK antisense ODNs corresponding to different regions of mouse MK coding sequence were synthesized. The sequence and location of each ODN in MK cDNA (Ref. 28; Fig. 1) were as follows:

antisense-1, 5'-AAGAAGCCTCGGTGCTGCAT-3' (bases 1 to 20);
AS, 5'-AGGGCGAGAAGGAAGAAG-3' (bases 15 to 32);
antisense-3, 5'-CTCCAAATTCCTTCTTCC-3' (bases 218 to 235); and
antisense-4, 5'-GGGCTTAGTCACGCGGAT-3' (bases 349 to 366).

Sense and reverse ODNs for AS were also designed as controls:
SEN, 5'-CTTCTTCCTTCTCGCCCTT-3'; and
REV, 5'-GAAGAAGGAAGAGCGGGA-3'.

Cell Culture Conditions and Transfection of ODNs. CMT-93 cells (American Type Culture Collection, Rockville, MD) derived from mouse rectal carcinoma were maintained in DMEM with 10% heat-inactivated FBS at 37 °C in a humidified atmosphere of 5% CO_2. Cells were inoculated at a density of 3×10^5 cells/35-mm tissue culture dish and cultured under the above conditions. After 15 h when cells reached to 70–80% confluence, they were transfected with ODNs in serum-free medium using LipofectAMINE-plus (Life Technologies, Inc., Grand Island, NY) in accordance with the manufacturer's instructions. Thus, 5 μl of each ODN stock solution (1 mM) and the plus reagent (10 μl) were mixed in DMEM (85 μl) in a small, sterile tube. After immediate mixing with a Vortex mixer and standing at room temperature for 15 min, the LipofectAMINE reagent (4 μl) in DMEM (100 μl) was added, and the mixture was left at room temperature for 15 min. Then, 0.8 ml of DMEM was added to generate ODN-transfection complex. Medium was removed from the culture of CMT-93 cells, and the ODN-transfection complex, the total volume of which was 1 ml, was added. After incubation for 3 h at 37 °C, 1 ml of DMEM with 10% FBS was added, and incubation was continued for 4 h. For proliferation analysis, colony formation in soft agar, and *in vivo* tumorigenicity study, the transfected cells were recovered by

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To whom requests for reprints should be addressed, at Department of Biochemistry, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan. Phone: 81-52-744-2089; Fax: 81-52-744-2065; E-mail: tmurama@med.nagoya-u.ac.jp.

[3] The abbreviations used are: ODN, oligodeoxynucleotide; MK, midkine; PTN, pleiotrophin; FBS, fetal bovine serum; BrdUrd, 5-bromodeoxyuridine; A. collagen, atelocollagen; SEN, sense control for AS; REV, reverse control for AS; AS, Antisense-2.

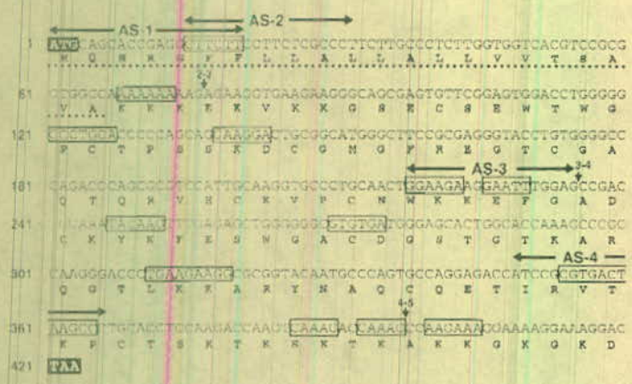

Fig. 1 Design of MK antisense ODNs. Locations of antisense-1, AS, antisense-3, and antisense-4 are shown by *horizontal arrows*. □, loop regions in the predicted secondary structure; *ATG* (*black box*), the translation initiation site; *TAA* (*black box*), the translation termination site; —, the signal peptide; *vertical arrows*, exon-intron junctions (number indicates exon number).

fixation and were used. For Western blotting analysis and ELISA, the medium was then replaced with fresh DMEM containing insulin (10 μg/ml), transferrin (5.5 μg/ml), sodium selenite (6.7 ng/ml), and heparin (20 μg/ml). After 16 h of incubation, conditioned medium was collected for analysis.

Proliferation Analysis. AS- and SEN-transfected CMT-93 cells were plated in 24-well plates in DMEM supplemented with 10% FBS at a density of 1×10^4 cells/well. Later (3 h), the medium was changed to serum-free medium, and the cells were cultured for ≤5 days. Cell proliferation was monitored using a cell counting kit (Dojin, Kumamoto, Japan). Chemically synthesized human MK (5 ng/ml; Peptide Institute, Osaka, Japan) was added to some AS-transfected cultures.

Colony Formation in Soft Agar. AS-, SEN-, or REV-transfected CMT-93 cells were suspended in 0.33% Noble agar (Sigma Chemical Co.) dissolved in 1 ml of serum-free medium and plated on top of a 1.5-ml base layer of 0.5% agar in the same medium in 35-mm dishes. After 9–11 days of incubation at 37°C in an incubator under a humidified 5% CO_2 atmosphere, the numbers of colonies formed in the whole dish were counted. Chemically synthesized MK (10 ng/ml or 50 ng/ml) was exogenously added as a rescue molecule to both base and top layers of some AS-transfected cultures.

In Vivo Tumorigenicity Study. A total of 1.9×10^5 AS- or SEN- (5 μM) pretransfected CMT-93 cells were s.c. inoculated in 0.3 ml of serum-free medium through a 24-gauge needle into both lower flanks of 8-week-old BALB/c nude mice obtained from SLC (Tokyo, Japan). One week after inoculation, AS or SEN plus A. collagen (Koken Co. Ltd., Tokyo, Japan) samples (total volume 50 μl/tumor) kept at 4°C were directly injected into the tumor region. The final concentration of A. collagen was 1.75% and that of each ODN was 40 μM. Tumor diameters were measured at regular intervals with calipers, and tumor volume in mm^3 was calculated by the formula: volume = (width)^2 × length/2 (29). Data are presented as means ± SE.

Tumor Therapy. A total of 4.5×10^5 untransfected CMT-93 cells were s.c. inoculated as described above. After 7 days when tumors reached an average volume of ~50 mm^3, the tumor-bearing nude mice were randomly divided into five different treatment groups (AS alone, SEN alone, AS plus A. collagen, SEN plus A. collagen, and PBS). AS or SEN with or without A. collagen was directly injected into the tumor region. The final concentration of A. collagen was 1.75% and that of each ODN was 50 μM. In positive controls, PBS alone was injected. Each therapeutic reagent was injected into the tumors every 2 weeks after the first injection. Animal experiments in the present study were performed in compliance with the guidelines of the Institute for Laboratory Animal Research, Nagoya University School of Medicine.

Western Blotting Analysis and ELISA. Proteins in conditioned medium were separated by electrophoresis on 15% SDS-PAGE gels and transferred electrophoretically onto nitrocellulose membranes (18). Blots were blocked with 5% nonfat dried milk and incubated with rabbit anti-MK antibody (18) and horseradish peroxidase-conjugated goat antirabbit IgG. Protein bands were visualized using an enhanced chemiluminescence detection kit (Amersham Pharmacia). Quantitative analysis of the blots was performed with an imaging densitometer. The amount of MK was determined by ELISA as described previously (30).

BrdUrd Treatment. For BrdUrd labeling, mice were injected i. p. with 200 μg of BrdUrd 16 h before sacrifice as described by Marzo *et al.* (7).

Immunohistochemistry. Parts of the tumor tissues were snap-frozen in liquid nitrogen and kept frozen at −80°C until use. Sections, 2-μm thick, were cut with a cryostat and fixed in acetone. Sections were then stained with rat antimouse CD31 antibody (PharMingen).

For BrdUrd immunostaining, the DNA of the tissue sections was denatured by incubation with 2 M hydrochloric acid for 10 min. To neutralize the pH, sections were rinsed in 0.1 M sodium tetraborate for 10 min followed by rinsing in PBS (31). The sections were then incubated with anti-BrdUrd rat monoclonal antibody (Oxford Biotechnologies Ltd.).

Specific antibody staining was visualized using FITC-labeled goat antirat IgG (Cappel, Durham, NC). The diluent of FITC-labeled secondary antibody was filtered (0.22 μm) before use to prevent nonspecific staining by contamination with FITC debris. After washing with PBS, all of the sections were mounted with a ProLong Antifade kit (Molecular Probes) and examined by confocal microscopy (MRC 1024; Bio-Rad).

Apoptotic cells were estimated using Apoptosis Detection kit (Takara Shuzo Co., Ltd., Shiga, Japan) based on the terminal deoxynucleotidyl transferase-mediated nick end-labeling method (32).

Intratumor Microvessel Density Assessment. Microvessel counting of CD31 (PECAM-1)-positive vessels was performed as described by Weidner *et al.* (33). To determine intratumor microvessel density, the five vascular areas within a section were selected and images were obtained with 200-fold magnification (microscope field: 666 μm × 666 μm, 0.444 mm^2) under a confocal microscope and computer system. Microvessels in each image of five areas were independently counted on a computer display, and the arithmetical mean in each area was used to calculate the mean intratumor microvessel density for each tumor section.

Statistical Analysis. The data were analyzed using the Mann-Whitney *U* test, and probability values <0.05 were considered to indicate significant differences.

RESULTS

Effects of MK Antisense ODNs on CMT-93 Carcinoma Cells in Culture. We predicted the secondary structure of mouse MK mRNA by computer analysis (Genetyx-Mac; Version 10); the algorithm for the calculation was that of Zuker and Stiegler (34). We designed MK antisense ODNs to target loop regions of the mRNA (Fig. 1). Of the four antisense ODNs synthesized, only AS suppressed synthesis and secretion of MK in CMT-93 rectal carcinoma cells after transfection with the aid of LipofectAMINE-plus (Fig. 2*A*). SEN and REV ODNs for AS showed no effects (Fig. 2*B*). Densitometric analysis of the blots revealed that 5 μM of AS suppressed MK production to 13% of

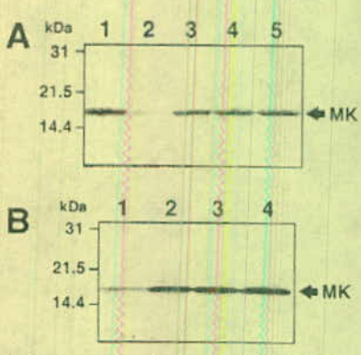

Fig. 2 Western blotting analysis of MK secretion in CMT-93 cells transfected with various ODNs. *A*, cells were transfected with 5 μM antisense-1 (*Lane 1*), AS (*Lane 2*), antisense-3 (*Lane 3*), antisense-4 (*Lane 4*), or no ODN (*Lane 5*), respectively. *B*, cells were transfected with 5 μM AS (*Lane 1*), SEN, (*Lane 2*), REV, (*Lane 3*), or no ODN (*Lane 4*). Conditioned medium were separated by SDS-PAGE, and the MK protein was detected with anti-MK antibody.

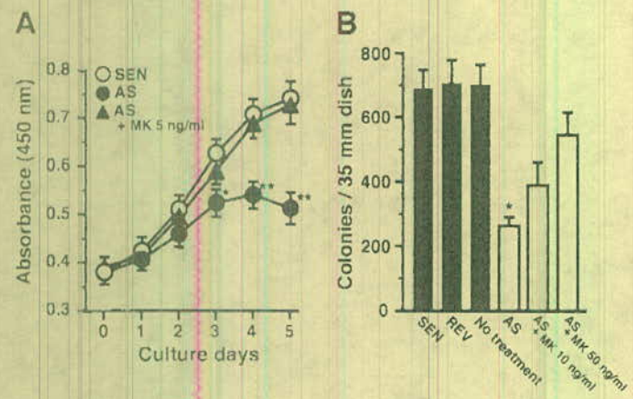

Fig. 3. Effects of AS on CMT-93 cells in culture. *A*, inhibition of cell proliferation. Cells were transfected with 5 μM AS (●) or 5 μM SEN (○) and assayed with a Cell Counting Kit every day for 5 days. Each plot represents the mean ($n = 4$), *bars*, ± SD. *, $P < 0.01$; **, $P < 0.001$ versus SEN. *B*, inhibition of anchorage-independent growth. AS-, SEN-, and REV-transfected cells were plated in 35-mm dishes at a density of 5000 cells/dish in agarose containing serum-free medium. Colonies were allowed to grow for 14 days and then counted. The results are expressed as means ($n = 5$), *bars*, ± SD. Exogenous MK for rescue was added to the AS-ODN-transfected culture as indicated in the figure. *, $P < 0.001$ versus SEN.

that in control cultures. ELISA estimation of MK in culture medium confirmed these results: the values were 218, 1439, and 1647 pg/ml for AS-, REV-, and SEN-, respectively.

When AS was added to cultures of CMT-93 cells, it inhibited their growth especially 4–5 days after addition. However, SEN showed no such effect (Fig. 3*A*). Addition of 5 ng/ml of MK abolished the effect of AS, confirming that the effect of AS was specific. Because the level of MK in the medium of untreated cells was about 1.5–1.8 ng/ml, the amount of added MK was not much excessive.

Colony formation in soft agar, a property closely associated with malignancy (35), was also suppressed by AS but not by SEN or REV (Fig. 3*B*). Exogenously added MK also counteracted AS on colony formation (Fig. 3*B*). That a higher concentration of MK (50 ng/ml) was required for the attenuation in this system may be explained by the interaction of MK with agar.

Effects of MK Antisense ODN on Tumor Growth in Nude Mice. Because AS specifically inhibited production of MK in CMT-93 cells and inhibited their growth and colony formation in soft agar, we examined the effects of AS on tumor formation by these cells. CMT-93 cells were treated with AS or SEN with the aid of LipofectAMINE-plus and were s.c. inoculated into male nude mice. After inoculation (7 days), tumor formation was clearly observed by SEN-pretreated cells, whereas barely visible tumor was formed by AS-pretreated cells (Fig. 4*A*). At that time, additional AS or SEN was injected with A. collagen but without LipofectAMINE-plus. In animals injected with SEN-transfected cells, massive and angiogenic solid tumors were observed, whereas the AS-transfected cells yielded much smaller tumors (Fig. 4*A*). This reduced tumor growth was dependent on the dose of AS injected (Fig. 4*B*), and 40-μM AS inhibited tumor growth by 80% as compared with SEN-treated cells (Fig. 4*B*).

Treatment of Preformed Tumors by MK Antisense ODN. We then injected preformed tumors with AS. Untreated CMT-93 cells were inoculated into nude mice. After inoculation (7 days) when a palpable tumor was formed, SEN or AS with or without A. collagen was injected into the tumor, and the injection was repeated every 14 days. LipofectAMINE-plus was not used in this series of experiments. AS with A. collagen markedly suppressed tumor growth as compared with SEN with A. collagen ($P < 0.001$; Fig. 5*A*). The rate of increase of tumor volume treated with AS with A. collagen was ~4.2-fold lower than that seen after treatment with SEN with A. collagen 10–41 days after initiation of tumor therapy (Fig. 5*A*). AS injection without A. collagen was less effective, although AS alone also delayed tumor growth as compared with SEN alone ($P < 0.01$; Fig. 5*A*). SEN with A. collagen scarcely inhibited tumor growth as compared with SEN alone or PBS injection (Fig. 5*A*), indicating that A. collagen alone does not have antitumorigenic activity to CMT-93 cells. This point was directly examined by injecting A. collagen to CMT-93 tumor (Fig. 5*B*). Indeed, the A. collagen-injected tumors exhibit growth properties indistinguishable from untreated tumors (Fig. 5*B*).

In the above experiment, tumor growth was monitored by determining tumor volume estimated with calipers. At the end of the study (41 days after initiation of tumor therapy), all of the animals were sacrificed, and tumor weight was determined. AS with A. collagen significantly suppressed the increase of tumor weight as compared with controls (Fig. 5*C*). Furthermore, we found that intratumor injection of AS with A. collagen resulted in significant reduction of tumoral MK content as determined by ELISA in accordance with their therapeutic effects (Fig. 5*D*).

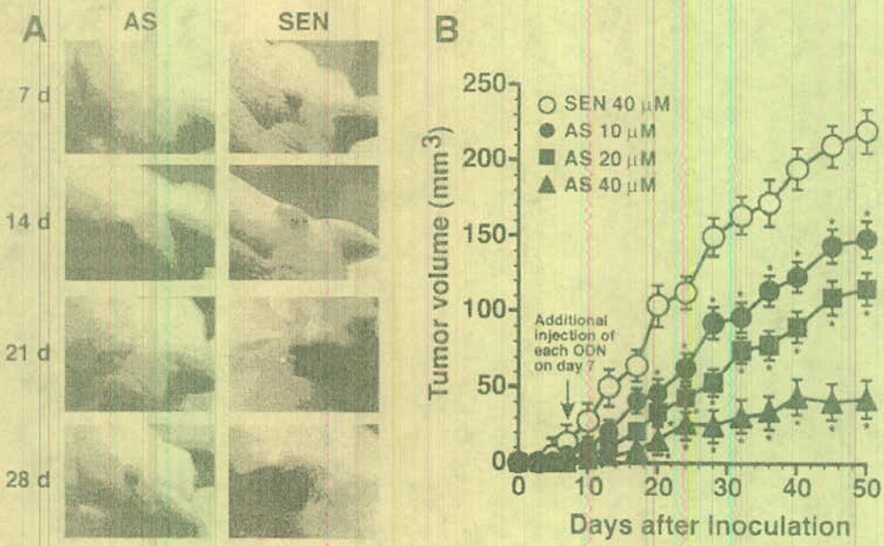

Fig. 4. Suppression of tumorigenesis of AS-transfected cells in vivo. A total of 9×10^6 AS- or SEN-transfected cells were s.c. inoculated into male nude mice (day 0). The concentration of ODN used for transfection was 5 μM. On day 7, SEN (40 μM) or AS (10, 20, and 40 μM for ●, ■, and ▲, respectively) mixed with A. collagen kept at 4°C was directly injected into the tumor region. Tumor diameters were measured at a regular interval for up to 50 days with calipers, and tumor volume was calculated. Each data point represents the mean ($n = 6$) of each group of mice. *Bars*, ± SE. *A*, $P < 0.001$ *versus* SEN (40 μM) with A. collagen.

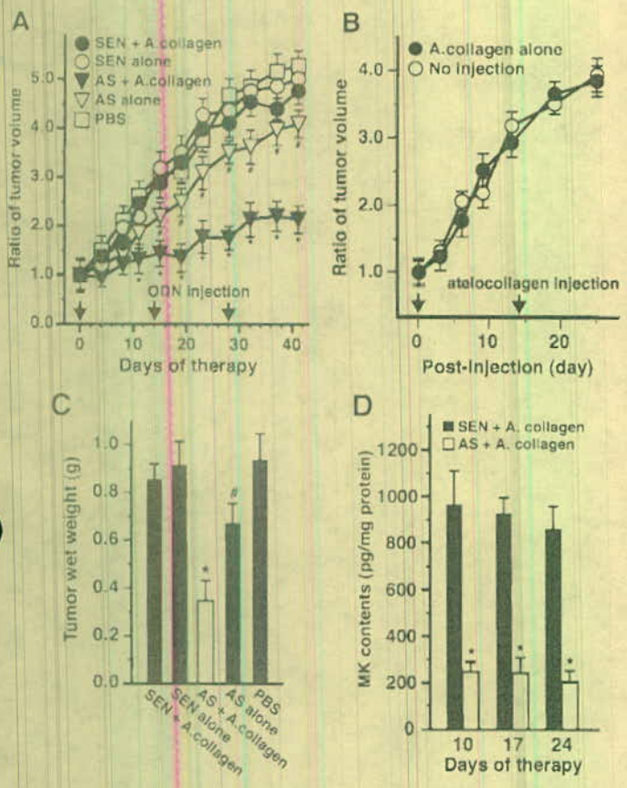

Fig. 5 Antitumor effect of AS in CMT-93 tumor-bearing nude mice. *A*, tumor growth was started on days 0, 14, and 28. AS (▲) or SEN (●) mixed with A. collagen, AS alone (▽), SEN alone (○), or PBS alone (□) was injected into the tumor region, as indicated in the figure. Days 0 correspond to 7 days after inoculation of cells, when tumor volume was ~50 mm³. Tumor diameters were measured at a regular interval for up to 41 days with calipers, and tumor volume was calculated. Results represent the means ($n = 6$). *bars*, ± SE. *, $P < 0.001$ *versus* SEN + A. collagen; #, $P < 0.01$ *versus* SEN alone. *B*, effect of A. collagen on tumor growth. On days 0 and 14, A. collagen alone (50 μl) was injected into the tumors (●). The controls were untreated tumors (○). Day 0 corresponds to 7 days after inoculation of CMT-93 cells. Tumor diameter was determined, and the result was expressed by ratio of tumor volume to that in day 0. Results represent the means ($n = 4$). *bars*, ± SE. *, tumor weights. Experiments were performed as in *A*. The mice were sacrificed 41 days after injection, all tumors were excised, and the tumor weights were determined. Results represent the means ($n = 6$). *bars*, ± SE. *, $P < 0.001$ *versus* SEN + A. collagen; #, $P < 0.01$ *versus* SEN alone. *D*, MK contents in tumors. Experiments were performed as in *A*. The excised tumors on days 10, 17, and 24 after therapy were homogenized in ice-cold Tris-buffered saline containing 1% Triton X-100 and protease inhibitor mixture (Sigma Chemical Co.) and then centrifuged. The amount of MK in each supernatant was measured by ELISA specific for MK. *, $P < 0.001$ *versus* SEN + A. collagen. Results represent the means ($n = 4$). *bars*, ± SD.

We evaluated whether AS exhibited antiangiogenic effects to suppress tumor growth *in vivo*. Ten and 17 days after initiation of therapy, intratumoral microvessel density was slightly but significantly suppressed by AS with A. collagen as compared with SEN with A. collagen (Fig. 6). On the other hand, cell division monitored immunohistochemically by incorporation of BrdU/rd was markedly suppressed by treatment with AS with A. collagen as compared with treatment with SEN with A. collagen (Fig. 7); the number of dividing cells in the former group was ~20% of that in the latter at both 10 and 17 days after initiation of therapy. The degree of suppression of cell division *in vivo* correlated well with the degree of suppression of tumor growth rate. However, no significant differences were observed in the degree of apoptosis determined by the terminal deoxynucleotidyl transferase-mediated nick end labeling method (32) between tumors treated with AS with A. collagen and those treated with SEN with A. collagen (data not shown).

DISCUSSION

An antisense ODN to MK successfully suppressed synthesis of MK in CMT-93 rectal carcinoma cells leading to inhibition of their growth in culture and colony formation in soft agar. Furthermore, tumor formation by the treated cells in nude mice was markedly inhibited. Even injection of the antisense ODN into preformed tumor tissue suppressed tumor growth significantly. These results clearly established that MK participates in growth of tumor cells both *in vitro* and *in vivo*. Although MK is known to be an angiogenic factor (36), angiogenesis in tumors treated with AS with A. collagen was only slightly inhibited in this occasion. In contrast, AS with A. collagen strongly inhibited cell division of the tumor cells *in vivo*. Thus, we considered that MK antisense ODN inhibited growth of the tumor principally by its anticell proliferation activity with some contribution of the antiangiogenic activity.

Treatment of pregrown tumors by injection with antisense or ribozyme reagents to growth factors or receptors has been successful in only a few cases (3, 7). Targeting of basic fibroblast growth factor and its receptor markedly suppressed melanoma growth in nude mice through prevention of angiogenesis (3). Growth of mesothelioma in nude mice was suppressed by transforming growth factor β-2 antisense ODN (7). The results of the present investigation strongly suggested that MK-directed therapy is a promising method for treatment of malignancy. The most attractive point of MK-targeted therapy is preferential expression of MK in tumors (12–17). The tumor-associated expression of MK also enabled the usage of the MK-promoter to mediate tumor-specific expression of suicide genes (37, 38). Serum MK level has recently been introduced as a tumor marker elevated in a wide variety of malignancies (39). Therefore, MK is a molecule with multiple implications in clinical oncology.

PTN, which has ~50% sequence identity with MK, has potent tumorigenic potential in NIH3T3 cells (40). Transfection with a ribozyme-cleaving PTN mRNA has been shown to suppress growth of pancreatic carcinoma, cholliocarcinoma, and melanoma (41–43), whereas treatment of preformed tumors with the ribozyme has not been reported. Recent studies have revealed components of signaling receptors for MK and PTN (24, 44–50), and the downstream signaling systems (19, 50–52). Much of the identified signaling molecules are shared between MK and PTN; they are receptor-like protein tyrosine phosphatase ζ (24, 46, 50), syndecans (44, 45), phosphatidylinositol 3′-kinase (17, 50, 51), and extracellular signal-regulated kinase (17, 50, 51). Because of the similar biological activities of MK and PTN (53) and their shared signaling molecules, simultaneous inhibition of

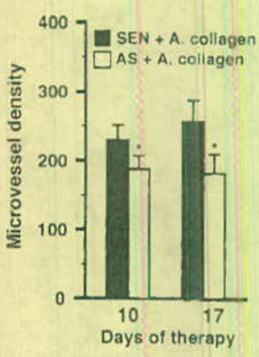

Fig. 6 Decreased vessel density in tumors injected with AS with A. collagen. Histological sections from tumors injected with AS or SEN mixed with A. collagen were immunostained for endothelial cells with anti-CD31 antibodies. We examined four excised tumors on both day 10 and 17 after therapy and intratumor microvessel density (vessels/mm²) was determined. Results represent the means ($n = 4$ tumors). *bars*, ± SD. *, $P < 0.05$ *versus* SEN + A. collagen.

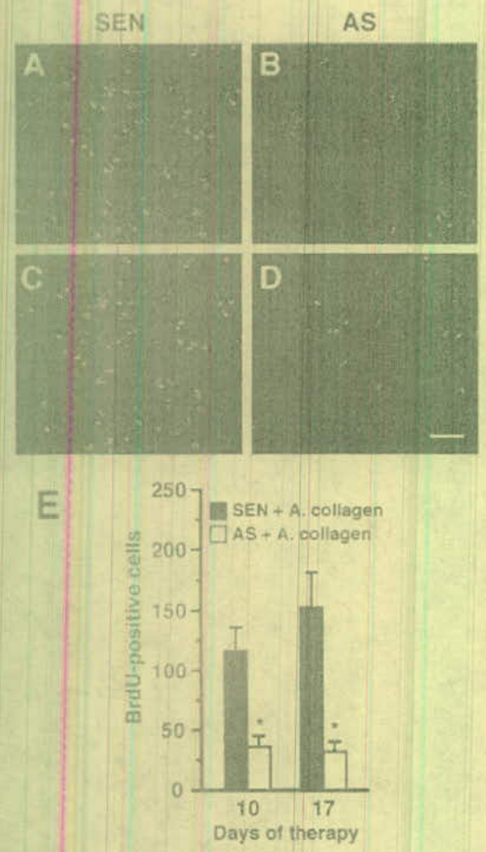

FIG. 7. BrdUrd immunostaining. Tumor-bearing mice were pulsed with BrdUrd 16 h before sacrifice to allow the uptake of BrdUrd by proliferating cells. Multiple tumor sections sampled at various time points were stained and analyzed by confocal microscopy. Tumor samples were from groups of mice injected with SEN (*A* and *C*) with A. collagen or AS (*B* and *D*) with A. collagen both 10 (*A* and *B*) and 17 (*C* and *D*) days after initiation of therapy. *Bar*, 50 μm. *E*, decrease in number of BrdUrd-positive cells in tumors injected with AS with A. collagen. Results (BrdUrd-positive cells/5 field at ×200) represent the means for 4 tumors/group. *Bars*, ± SD. *, $P < 0.001$ *versus* SEN + A. collagen.

expression of both factors may be required when they are coexpressed in the tumor. Because CMT-93 cells used here did not express PTN,[4] these cells are a suitable system in which to evaluate the effects of MK-directed therapy without consideration of PTN.

Successful treatment of tumors with MK antisense ODN was achieved not only by selecting a suitable molecule, MK, but also by choosing suitable delivery conditions using A. collagen. A. collagen has been found to be effective in delivery of antisense ODN directed to ornithine decarboxylase, and the antisense treatment suppressed growth of a number of tumor cells *in vivo*.[5] A. collagen is unique in that it is liquid at 4 °C and a gel at 37 °C. Furthermore, A. collagen shows neither antigenicity nor toxicity in animals, because antigenic telopeptides attached to both ends of the collagen are eliminated by pepsin digestion (26). We consider that addition of A. collagen resulted in increased cellular uptake, nuclease resistance, and prolonged release of MK antisense ODN, as in the case of plasmid DNA (26). A. collagen forms ionic complex with ODN; this phenomenon is regarded as the basis of the above-mentioned action of A. collagen.[6]

It has been reported that phosphorothioate ODNs interact and regulate the heparin-binding proteins including some growth factors and that the interaction is sometimes sequence specific (54). Contribution of such a mechanism to effects of antisense MK ODN is probably low, if any, because antisense MK ODN other than phosphorothioate ODN exhibited similar activities,[7] and antisense MK ODN was considered to be slowly released and delivered to tumor cells, probably as a complex with A. collagen. However, occurrence of such action of phosphorothioate ODN (54) should be taken into account in future studies.

In conclusion, we demonstrated potent growth inhibitory effects of MK antisense ODN on rectal carcinoma growth *in vivo*. Because strong expression of MK is not observed frequently in adult tissue (8, 12–17), MK-directed therapy is expected to show stringent tumor specificity with low side effects. Much work remains to be performed before MK antisense ODN can be applied to treatment of human cancer. Specifically, tumor suppressive effects of the reagent should be examined in other types of tumors to define the scope of MK-directed therapy.

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Oligo oligarchy—the surprisingly small world of aptamers

Has skepticism about nucleic acid therapeutics shaped the competitive landscape for emerging aptamer companies? Karl Thiel reports.

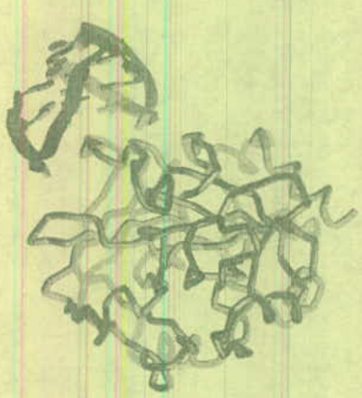

Figure 1 The thrombin aptamer (aqua) forms a specific binding surface with the thrombin protein (blue).

After a hiatus of over two years, the biotech initial public offering window is open—but with a different focus. Where investors once threw cash at companies with platform technologies but no products or foreseeable prospects of profits, now they want late-stage products addressing large audiences and unmet medical needs. But amid this conservative focus there has been an appetite for something a little racier—aptamers, oligonucleotide-based therapeutics that directly bind proteins. Three of 21 companies to go public since last October—Eyetech (New York), Dynavax (Berkeley, CA, USA) and Corgentech (S. San Francisco, CA, USA)—work on this largely unproven modality. A fourth company, Archemix (Cambridge, MA, USA), raised $50 million in April—an IPO-like sum in this market. All this, despite the fact that these drugs are chemically similar to two largely failed modalities of the past, antisense and ribozymes.

Don't expect a slew more aptamer companies to earn their tickers anytime soon, however. As remarkable as is the interest in this therapeutic strategy is the concentration of intellectual property (IP) assets into just a few players that appear to have successfully blocked most competitive threats. (See Table 1 for a list of companies.)

Third time the charm?

Aptamers are relatively short (usually 12–30 base) single-stranded oligonucleotides that assume specific, stable conformations *in vivo* and bind tightly to very specific protein targets. In terms of chemical composition, these nucleic acid ligands are essentially identical to antisense drugs—indeed, RNA aptamers have been modified in some of the same ways as antisense molecules to avoid problems of rapid nuclease degradation in the bloodstream and short serum half-life. But because they target proteins, aptamers, unlike antisense drugs, can work against either intra- or extracellular targets—obviating the need, at least for some applications, to get the molecules inside of cells.

Yet whereas antisense and another nucleic acid–based therapeutic technology, ribozymes, have brought investors more heartache than success, the reception for aptamers remains enthusiastic. (The most recent setback for antisense came on May 3, when an FDA panel rejected Genasense, an antisense drug for malignant melanoma developed by Genta (Berkeley Heights, NJ, USA) and Aventis (Strasbourg, France), that produced equivocal clinical results.)

How did aptamers, an even younger nucleic acid–based modality, gain the confidence of twice-shy investors? The ambassador for this new kind of therapeutic is a drug called Macugen (pegaptanib sodium), being developed by Eyetech for the treatment of the 'wet' forms of age-related macular degeneration (AMD). Although many companies that became newly public in 2003 brought disappointing returns to investors, Eyetech brought in a one-day return of 54% and has been the ninth-best-performing IPO in the past 12 months across all industries. Despite the fact that Macugen is Eyetech's only product—there is nothing else in the company's pipeline even remotely close to the clinic—Eyetech commands a market cap north of $1.4 billion.

That rich valuation can be attributed to some very robust results in interim analyses of two pivotal phase 2/3 trials, and to the huge potential market for Macugen—some 1.6 million cases of wet AMD in the United States alone, with an estimated 200,000 new cases each year, a figure likely to grow as our population ages. A sweetheart deal the company landed with Pfizer (Groton, CT, USA) didn't hurt either—it brings Eyetech up to $750 million in various payments and still leaves the young company an equal share of all profits. Eyetech intends to submit a licensing application to the FDA in the third quarter of this year, and with an accelerated approval agreement from the agency, Macugen stands a very good chance of reaching the public in early 2005.

Class distinction

What is more exciting to some venture capitalists than this single product, however, is the virtues of aptamers as a therapeutic class to treat any number of illnesses. In theory, these molecules offer some of the best of both small molecules and monoclonal antibodies.

On the small-molecule side of the equation is the stability and seeming lack of immunogenicity of aptamers. Anthony Adamis, Eyetech's CSO and cofounder, describes them as "rock stable."

"You can heat them up to 80 or 90 degrees [Celsius], cool them down, and they will still work," he says. "They can survive in rather harsh environments with various solvents." Adamis believes the natural stability of aptamers will eventually help his company develop an extended release or alternative delivery formulation of Macugen, which was given by intravitreal injection every six weeks in clinical trials. In addition, no evidence of antibody development or other immunogenicity to Macugen was observed in clinical trials, he says—a claim echoed by others who have used aptamer therapeutics in the clinic.

Aptamers may also share some common ground with small molecules when it comes to manufacturing. Making Macugen, says Adamis, "is a straightforward 15-step synthetic process. Unlike an antibody, where you have to build a gigantic fermentation plant, this is straightforward chemical synthesis." Archemix president and CEO Errol De Souza adds that the synthetic process is also relatively free of patent and royalty entanglements that surround antibody production.

On the antibody side of the equation is the specificity and binding affinity of aptamers. Aptamers have a larger surface area in comparison to small molecules, offering more topological and chemical points of interaction with targets, and thus antibody-

like dissociation constants (Fig. 1). In terms of specificity, Adamis notes, "aptamers have been created that have a 10,000-fold greater affinity for caffeine versus theophylline—and these are molecules that differ only by a methyl group." Macugen, for instance, binds to only one of six isoforms (isoform 165) of vascular endothelial growth factor (VEGF), a specificity that Adamis argues makes the drug safer and more effective than a complete VEGF blockade.

Aptamer oligopoly

With these traits in mind, some investors are betting that aptamers will find a significant niche in tomorrow's pharmacopeia. And unlike antibodies, where entrepreneurs must brave a very competitive landscape, the intellectual property (IP) rights surrounding aptamers are remarkably centralized.

That concentration can be attributed in large part to Larry Gold, one of the developers of an *in vivo* process for selecting aptamers out of large combinatorial pools of oligonucleotides, called SELEX[1] (for systematic evolution of ligands by exponential enrichment). Gold, along with venture capitalist Patrick Mahaffy, founded NeXagen (Boulder, CO, USA) in 1991 to exploit this technology. The company later merged with Vestar to become NeXstar, which was in turn acquired by Gilead Sciences (Foster City, CA, USA) in 1999.

Although a decade of work went into aptamers, including the development of Macugen (then called NX-1838) as far as a phase 1 trial, Gilead's management decided to focus the business around the company's antiviral drug franchises, and to divest both the aptamer and oncology assets of the company. Gold has since gone on to found SomaLogic (Boulder, CO, USA), which is focused on diagnostic applications of aptamers.

Eyetech licensed exclusive rights to Macugen from Gilead in 2000—indeed, the company was formed specifically to take advantage of that licensing opportunity. In October 2001, the rest of Gilead's aptamer assets went to Archemix, with Gilead receiving $17.5 million plus warrants in Archemix.

Using the SELEX process, to which Archemix now owns the exclusive rights for discovering therapeutic molecules, researchers are able to screen huge libraries on the order of 10^{15}–10^{16} unique oligonucleotides against their targets. The SELEX estate includes not just this discovery process, however, but over 175 patents on specific compositions and methods of use—including claims as broad as "a non-naturally occurring nucleic acid ligand having a specific binding affinity for a target molecule, said target molecule being a protein, wherein said nucleic acid ligand is not a nucleic acid having the known physiological function of being bound by the target molecule"[2]. In other words, they have rights to any nucleic acid sequence that binds to any protein—the entire universe of synthetic aptamers—as long as it doesn't involve a known natural process, such as the interaction between transcription factors and DNA.

One key way in which aptamers differ from both small molecules and antibodies is in the potential universe of drug candidates. With potential therapeutics limited to oligonucleotides ranging from about 10 to 40 bases in length, there is a finite number of molecules to be screened—a stark contrast to combinatorial synthesis of small molecule libraries, where even the largest collection represents an infinitesimal piece of conceivable chemical space.

The three-dimensional diversity of aptamers is somewhat smaller than potential sequence diversity, suggests Archemix executive vice president Martin Stanton. Reversing

Table 1 Therapeutic aptamers in clinical development

Company (partner)	Drug (indication)	Status
Aptamera (Louisville, KY, USA)	Agro100 (cancer)	Phase 1 launched September 2003
Archemix (Nuvelo) (Lyon, France)	ARC-183 (short half-life anticoagulant/antithrombotic for CABG)	Phase 1 planned for second half of 2004
Coley Pharmaceutical Group (Wellesley, MA, USA)	ProMune (non-small-cell lung cancer, melanoma, cutaneous T-cell lymphoma)	Phase 2; phase 3 trial planned for late 2004. Phase 1/2 data available late 2004; phase 2 planned for second half 2004
	Actilon (hepatitis C)	Phase 1—development done by partners
(GlaxoSmithKline and Chiron)	VaxImmune adjuvant (cancer)	Two products scheduled to enter phase 1 in 2004
(Aventis)	Asthma and allergy compounds	
Corgentech (S. San Francisco, CA, USA) (Bristol-Myers Squibb)	E2F decoy (to prevent vein graft failure)	Enrollment complete in two phase 3 trials. Phase 1/2 trial for arteriovenous graft failure planned for first half of 2004.
Dynavax (Berkeley, CA, USA) (UCB Farchim)	AIC-1018 ISS linked to ragweed allergen (ragweed)	Phase 2/3 trial launched February 2004.
	1018 ISS as adjuvant mixed with hepatitis B allergen as prophylaxis. (A-linked version for therapeutic use is in preclinical development)	Phase 3 trial planned for 2004.
(Berna Biotech)	1018 ISS inhalation (asthma)	Phase 2, results in mid-2004
Eyetech (Pfizer) (New York, NY, USA)	Macugen (all forms of wet AMD)	Interim results of two pivotal phase 2/3 trials reported; NDA filing planned for third quarter 2004
(Pfizer)	Macugen (diabetic macular edema)	Phase 2 trials ongoing
(Pfizer)	Macugen (retinal vein occlusion)	Phase 2 trial planned for 2004
Hybridon (San Diego, CA, USA)	HYB2055 (cancer)	Phase 2 planned for summer 2004

AMD, age-related macular degeneration; CABG, coronary artery bypass grafting; NDA, new drug application.

a C and G in the structural portion of the molecule, for instance, does not appear to make a difference to the final shape of the molecule. Thus, a library of 10^{15} oligos is a pretty good-sized chunk of the practical aptamer universe. Using SELEX, Archemix researchers are able to get to success or failure more quickly than they might with conventional small molecule screening.

Backdoor into cells

Other interactions between nucleic acid ligands and proteins that have "known physiological function" would hence presumably fall outside of Archemix's broad net. Three companies—one of them freshly public this year—are working on aptamers that target Toll-like receptor 9 (TLR9) on dendritic cells (Fig. 2). This receptor appears to provide a solution to what is otherwise a drawback of aptamers—they don't get into cells very well. In fact, it appears that TLR9 evolved specifically to bring certain nucleic acids into intracellular compartments called endosomes.

In the genomes of vertebrates, cytosine and guanine (C and G) seldom occur together in sequence—and when they do, the cytosine is usually methylated. Unmethylated CpG dinucleotides, however (where the p represents a phosphate bond), occur frequently in bacterial genomes. It is now widely believed that humans evolved a mechanism to recognize CpGs as signaling a foreign invader, and TLR9 is the gateway by which this foreign genetic information is taken into cells and used to mount an immune response.

This led researchers at both the University of Iowa (Iowa City, IA, USA) and the University of California-San Diego (UCSD) to explore the idea of using CpG-containing immunostimulatory sequences as therapeutics. The resulting UCSD patents were licensed to Dynavax (Berkeley, CA, USA), whereas Arthur Krieg, who while at the University of Iowa made a key discovery on the immunostimulatory role of CpGs[3], went on to found Coley Pharmaceutical Group (Wellesley, MA, USA). Currently a patent dispute rages between the two companies concerning the priority of early inventions in the field, but Dynavax and Coley are actually using the technology to somewhat different ends—Dynavax, with a primary focus on allergy, asthma and hepatitis B, Coley with a focus on cancer vaccines. Hybridon (Cambridge, MA, USA) is also working on aptamers to TLR9, but has developed a compound using synthetic immunostimulatory motifs instead of CpGs, which may put them outside the IP battle surrounding Dynavax and Coley.

Smooth sailing

With the exception of this one patent dispute, the aptamer space is remarkably placid. Because of Archemix's broad ownership of IP rights in the field, there are relatively few competitors in the space and virtually no legal wrangling. It is probably impossible to tell whether the concentration of IP in one company is in any way slowing the potential development of aptamer drugs, but Archemix has thus far been willing to grant licenses to other companies seeking to develop aptamer drugs. "Licensing is at least a portion of their business model," says Chris Rusconi, vice president of discovery and development at Regado Biosciences (Morrisville, NC, USA), a young company working on "antidote-controlled" aptamers, where a therapeutic effect can be turned off by introducing a sequence complementary to a portion of the therapeutic sequence. "Do they want to go and enable a thousand little competitors? I don't think so. But they've contemplated this and they've been a very good corporate partner."

Archemix has likewise granted a license to Aptamera (Louisville, KY, USA), which is developing an aptamer that targets nucleolin, a target found on the surface of many kinds of cancer cells. In both these cases, Archemix has kept a stake in any resulting commercial drugs; they have also granted a license to Noxxon Pharma (Berlin) that does not bear royalties. Noxxon is developing 'Speigelmers,' L-form mirror-image aptamers that have greater stability *in vivo* than unmodified RNA[4]. Other aptamers in clinical development have achieved stability against nuclease degradation through a bag of tricks used with antisense drugs—2-OH modifications of ribose and 3′ 'end caps' to block exonuclease activity, or modifications of backbone linkage or bases.

Overcoming obstacles

There are a few drawbacks to aptamers. In terms of manufacturing cost, they are much more like antibodies than small molecules. Eyetech isn't revealing manufacturing costs for Macugen, but Archemix's Stanton estimates that his company's first clinical candidate, an anticoagulant called ARC-183, will cost $50 or less per gram at manufacturing scale. Since aptamers have a lower molecular weight than antibodies but similar binding affinity, they should cost far less than antibodies on a per dose basis (1 gram of Macugen yields 3,000 doses, notes Eyetech's Adamis), but still much more than small molecules.

A more serious limitation of aptamers is familiar to those who have worked with other nucleic acid therapeutics: they do not, under normal circumstances, get inside of cells. "Aptamers get in as well or as badly as antisense or siRNA," acknowledges De Souza, but since they target proteins instead of message, drug developers have more flexibility in selecting their targets. "As part of our business strategy, we've said in the first phase of our business plan that all of our targets will be extracellular—just because we do not want to struggle with the cell barrier that antisense has struggled with."

Perhaps the most limiting drawback has to do with serum half-life. Unlike antibodies, which can linger in the bloodstream for days or even weeks, aptamers are quickly eliminated by the kidneys. ARC-183, deliberately designed for a short duration of action, has a half-life of about 2 minutes. This can be increased by attaching polyethylene glycol (PEG) molecules to the oligonucleotides—Macugen, for instance, is linked to two branching 20 kDa PEGs—but there are limits to what this can achieve. The longest half-life Archemix has achieved for any aptamer is just over 24 hours, although Stanton notes that this result is from rats and should double or more in humans. De Souza argues that the ability to "dial up and down" half-life with different size PEGs can be an advantage, but it appears that the flexibility is for now somewhat limited on the upper end.

Nevertheless, if Eyetech gains US marketing approval for Macugen in 2005, it will be a huge vindication for aptamers as a therapeutic class—one that antisense and ribozymes have never really earned—and proof that Eyetech's founders made a shrewd gamble by licensing Macugen when it was in phase 1. "Eyetech took a risk on a whole new therapeutic modality," says De Souza. With the current enthusiasm surrounding this class of molecules, it may no longer be clear how risky that decision seemed a few years ago. Eyetech's success will probably benefit Archemix as well. "We founded this company on the belief that aptamers are underappreciated and ready to be commercialized," says Stanton. "It was a bit of a leap at the time, and I don't think it was widely appreciated."

Karl Thiel is a freelance writer based in Portland, Oregon, USA.

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