The Generation and Characterization of Antagonist RNA Aptamers to Human Oncostatin M*

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Andrew Rhodes‡§, Angela Deakin‡, John Spaul§, Barry Coomber**, Alan Aitken‡‡, and Stephen Rees‡

From the ‡Molecular Discovery Department, the §In Vitro Pharmacology Department, the ¶Comparative Pathology Department, the **Chemical and Analytical Technologies Department, and the ¶¶Molecular Pharmacology Department, Glaxo Wellcome Research and Development, Gunnels Wood Road, Stevenage, Hertfordshire SG1 2NY, United Kingdom

Oncostatin M (OSM) is a multifunctional member of the interleukin-6 cytokine family. OSM has been implicated as a powerful proinflammatory mediator and may represent a potentially important, novel therapeutic opportunity for treatment of established rheumatoid arthritis. To further investigate the role of OSM in inflammatory disorders, we have isolated a series of RNA aptamers that bind specifically to human OSM. The highest affinity aptamer, designated ADR58, has been characterized in a series of in vitro and cell based assays. ADR58 has an affinity of 7 nM for human OSM, and it can antagonize OSM binding to the gp130 receptor and specifically antagonize OSM mediated signaling. The aptamer has been truncated in length to 53 bases, all pyrimidine positions are substituted with 2′-fluorine, and 14 of 18 purine positions have been substituted with 2′-O-methyl to increase stability toward nucleases. This truncated, modified form of ADR58 retains complete affinity and functional activity for OSM. This aptamer may be used as a tool to further investigate the role of OSM in inflammatory disorders and may also have role as a therapeutic agent.

Rheumatoid arthritis is a chronic inflammatory disease of articular joints, characterized by synovial hyperplasia and extensive cellular infiltration by mononuclear cells and polymorphonuclear leukocytes. A complex and poorly understood interplay between resident and infiltrating cell types leads to the chronic secretion of metalloproteinases, resulting in the destruction of articular cartilage, ligaments, and subchondral bone (1). In recent years, considerable evidence has emerged that indicates this fact. It may be a powerful mediator of the pathology of rheumatoid arthritis. OSM (2) is a 28-kDa glycoprotein that belongs to a family of cytokines comprising interleukin 6 (IL-6), IL-11, leukemia inhibitory factor (LIF), ciliary neurotrophic factor, and cardiac trophin 1 (3). The receptors for all family members share a common signal chain, gp130, as part of a complex family of hetero- and homodimeric receptors (4). OSM shares a common heterodimeric receptor with LIF (LIF receptor: gp130, type I) and also has its own unique receptor comprising OSM receptor β chain and gp130 type II (5). OSM has long been known to have effects on cell growth and differentiation (6). OSM has also been shown to have potent proinflammatory properties in mice (7) and demonstrates potent synergy with interleukin-1 to promote articular cartilage degradation in models systems ex vivo (8). OSM induces a prolonged increase in P-selectin in endothelial cells (9), stimulates urokinase-type plasminogen activator activity in human synovial fibroblasts (10), and is a powerful inducer of IL-6 from endothelial cells (11). OSM has recently been measured in rheumatoid arthritis but not osteoarthritis synovial fluid (12, 13) or synovium, production of which has been localized to macrophages (14). Mouse OSM has been shown to induce tissue inhibitor of metalloproteinase-1 in mouse fibroblasts and will induce acute-phase protein production by rat hepatocytes in vitro (15). It has also been shown that mouse OSM can induce both local and systemic effects in vivo (16). Amelioration of arthritis in two murine models has been demonstrated using antibodies to OSM (17).

The systematic evolution of ligands by exponential enrichment (SELEX) procedure is a protocol in which single stranded oligonucleotides are selected from vast libraries of sequences, based on a desired activity at a target protein or other molecule (18–21). The SELEX procedure is usually initiated with an RNA or DNA library consisting of some 10¹⁴-10¹⁵ random oligonucleotide sequences. In a fully randomized oligonucleotide library, each molecule will exhibit a unique tertiary structure that will be dependent on the nucleotide sequence of that molecule. The binding affinity of the oligonucleotide for that protein will be determined by the fit between moieties on the surface of the oligonucleotide and epitopes on the target protein. As a consequence of starting from a library of vast diversity, it is often possible to identify aptamers of nanomolar or subnanomolar affinity for the target protein with selectivity for that target protein over other proteins with a high degree of structural homology (18–21). Using SELEX methodology, RNA or DNA aptamers have been generated to many proteins, peptides, and small molecules, including dopamine (22), substance P (23), subtilisin (24), platelet derived growth factor (25), vascular endothelial growth factor (26), thrombin (27), and L-selectin (28).

Aptamers have been demonstrated to have biological activity, both in vitro and in vivo. For example, an RNA aptamer to the bacterial protease subtilisin was generated (24). The aptamer was able to block the protease activity of subtilisin using a small chromogenic peptide and showed no inhibitory activity to trypsin or chymotrypsin. In a second example high affinity DNA aptamers have been raised against platelet-derived growth factor and used to investigate the role of platelet-
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derived growth factor in renal disease (29). The modified aptamer was evaluated in a rat mesangioproliferative glomerulonephritis model. The aptamer lead to a 95% reduction in SCHMK buffer (110 mM NaCl, 1 mM
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pared by
fied RNA containing a randomized region of 40 nucleotides was pre-
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maturation (2) Following these manipulations, human OSM was ex-
pressed as a glutathione S-transferase fusion protein in Escherichia
coli. Following purification, OSM was cleaved from glutathione S-transferase by treatment with thrombin, and OSM was further purified from the glutathione S-transferase moiety.

SELEX—Iterative rounds of selection/amplification were performed as described (30) except that protein was bound to a 96-well plate for the partition phase of the SELEX process (36). 2-Fluoropyrimidine-modif-
ied RNA containing a randomized region of 40 nucleotides was pre-
pared in vitro transcription from synthetic random DNA templates. Template DNA (5′-ggggaggagccgg-40N-ccgagctgcctec5′), design-
ated 40N7, was prepared on an automated solid-phase synthesizer
(Applied Biosystems) according to the manufacturer’s protocol.

For the SELEX experiment, human OSM at 3 μg/ml was diluted to
the required concentration in SCHMK buffer (110 mM NaCl, 1 mM
HPEES, pH 7.0, 1 mM CaCl2, 5 mM KCl) and incubated in a
96-well microtiter plate (Labsystems) overnight at 4 °C to allow plate binding. The solution was removed from each well, and 200 μl of
block buffer (0.1% 1-Block (Tropix) in SCHMK buffer) was added. The plate was incubated at room temperature for 1 h. RNA was diluted in 200
μl of wash buffer (0.1% 1-Block, 0.05% Tween 20 in SCHMK buffer),
was added to individual wells at the concentrations shown in Table I. The plate was incubated at 37 °C for 30 min, after which, individual wells
were washed six times (200 μl each) with wash buffer at 37 °C to
remove unbound RNA. To elute specifically bound RNA 50 μl of water
was added to individual wells, and the plate was heated at 95 °C for
10 min. Reverse transcription was then carried out in individual wells,
followed by polymerase chain reaction and transcription to generate
RNA for the next round of SELEX as described previously (30).

Dissociation Constant Measurements—Binding assays were carried
out by nitrocellulose filter partitioning as described (30, 31).

Cloning and Sequencing—Aptamer DNA was cloned using the TA cloning kit (Invitrogen) in the plasmid pCR2.1 according to the
manufacturer’s instructions. Plasmid clones were sequenced according to a
polymerase chain reaction sequencing protocol (32).

Synthesis of Truncated Aptamers—Aptamers were synthesized on an
Applied Biosystems 394 oligonucleotide synthesizer according to opti-
mized protocols. Controlled pore glass functionalized with the appro-
riate protected ribonucleoside or with 3′-phosphoramidites, and 2′-OMe purine ribonucleotide phos-
phoramidites were also
purchased from Proligo. After deprotection using standard conditions for ribo-oligonucleotides, purification was achieved by anion-exchange high performance liquid chromatography using a Dionex DNA PacTM PA-100 column. The purified aptamers were characterized by negative ion electrospray mass spectrometry.

OSM-gp130 ELISA—Nunc immunoplates (F6 Maxisorp) were
coated overnight (4 °C) with human OSM (50 μg/ml, 1 μg/ml in carbonate/
bicarbonate buffer, pH 9.4). Plates were washed (six times in phosphate-buffered saline (PBS) 0.05% Tween 20, using Skatron plate washer), tapped dry, and blocked to reduce nonspecific binding (200
μg/ml, 1% BSA/PBS). Following a 1-h incubation (room temperature
on a shaking platform), the plates were washed (six times) and developed using an ELISA amplification system (Life Technologies, Inc.) according to the manufacturer’s instructions, and the absorbance was measured at 490 nm. On each plate, the total binding was determined by gp130-Fc conjugate and OSM in the presence of BSA/SCHMK, and nonspecific binding was determined by gp130-Fc conjugate in absence of OSM or conjugate binding to OSM in absence of gp130-Fc. The gp130 control ELISA was carried out as for the OSM-gp130 ELISA except that plates were coated with gp130 (50 μg/ml, 200 ng/ml in carbonate/bicarbonate buffer, pH 9.4).

TNF-TNF Receptor 1 ELISA—Nunc immunoplates (F6 Maxisorp) were coated overnight (4 °C) with sheep anti-human IgG (50 μg/ml, 1 μg/ml in PBS). Plates were washed (six times in PBS 0.05% Tween 20, using Skatron plate washer), tapped dry, and blocked for 1 h (room temperature on a shaking platform) to reduce nonspecific binding (200
μg/ml, 1% BSA in PBS). Plates were washed as above, and TNF receptor 1 monoclonal antibody (Clone: 1D7, purchased from Glaxo Wellcome). Following a further 1-h incubation (room temperature on a shaking platform), the plates were washed as above, and aptamer was added (50 μg/ml, 0.34 μg/20 μg, titrated in SCHMK buffer). As a control positive, an anti-TNF receptor 1 monoclonal antibody (R&D Systems) was included. Following a 2-h incubation (room temperature on a shaking platform), a complex of biotin-TNF-α (1 ng/ml, NEN Life Science Products) and streptavidin-alkaline phosphatase conjugate (1: 1000, Amersham Pharmacia Biotech) in 2% BSA/SCHMK buffer (50 μg/ml) was added. Following a 2-h incubation (room temperature on a shaking platform), the plates were washed (six times) and developed using ELISA amplification system (Life Technologies, Inc.) according to the manufacturer’s instructions, and the absorbance was measured at 490 nm. On each plate, the total binding was determined by TNF receptor 1 and biotin-TNF-α conjugate in the presence of BSA/SCHMK buffer and nonspecific binding of biotin-TNF-α conjugate in absence of the receptor or binding of the conjugate to the receptor in the absence of biotin-TNF-α.

HepG2 B6 Assay—A HepG2 (HepG2B6) cell line stably transfected with a reporter gene containing six functional signal transduction and five activator of transcription 3 response elements upstream of a secreted alkaline phosphate reporter was previously generated at Glaxo Wellcome.2 HepG2B6 cells were maintained in Dulbecco’s modi-
ified Eagle’s medium, 10% heat-inactivated fetal calf serum, 2 mM glut-
tamine, 1% nonessential amino acids, 500 μg/ml G418 (growth medium,
37 °C in 5% CO2 and 92% humidity. For assay, cells were plated into 96-well plates to a final concentration of 3 × 104 cells/well in 100 μl of growth medium containing 10% heat-inactivated fetal calf serum. Cells were allowed to equilibrate for 48 h. Anti-OSM aptamer was
diluted in 2 ng/ml OSN or 4 ng/ml IL-6 and incubated for 2 h at
37 °C. Dilutions were made in HepG2 B6 medium with 1% heat-inac-
tivated fetal calf serum. The old medium was removed from the wells
and replaced with 100 μl/well of aptamer/OSM mixture, and cells were incubated for a further 20 h. Each dilution was performed in triplicate.

Cells were allowed to equilibrate for 2 h at 37 °C. Dilutions were made in HepG2 B6 medium with 1% heat-inac-
tivated fetal calf serum. The old medium was removed from the wells
and replaced with 100 μl/well of aptamer/OSM mixture, and cells were incubated for a further 20 h. Each dilution was performed in triplicate.

Assay for IL-6 Release from KB Cells—KB cells (ECACC NO
94050408) were plated into 96-well plates to a final concentration of 2 × 104 cells/well in 100 μl of medium (Dulbecco’s modified Eagle’s medium, 10% heat-inactivated fetal calf serum (Sigma), 2 μg/ml human IL-6, and streptovitcin) and allowed to adhere overnight. Aptamers were incubated with human OSM or LIF (R&D Systems, carrier-
free) for 2 h at 37 °C in KB cell medium prior to addition to the cell monolayer. Following a 24-h incubation at 37 °C, the supernatants were harvested and stored at −20 °C for subsequent IL-6 measurement by ELISA (using antibody pairs from R&D Systems as described briefly

2 M. Saunders, unpublished observations.
RESULTS

Human OSM with both the N-terminal 25-amino acid leader sequence and the C-terminal 33 amino acids removed was expressed and purified as a glutathione S-transferase fusion protein from E. coli. Following purification, the integrity of the expressed OSM was confirmed by SDS-polyacrylamide gel electrophoresis (data not shown). To demonstrate that purified human OSM retains biological activity, the ability of this protein to stimulate secreted placental alkaline phosphatase expression in HepG2B6 cells was examined. The purified protein was shown to be equipotent to recombinant human OSM purchased from R&D Systems in this assay (data not shown). In competition experiments performed in the same cell line using both anti-human OSM and anti-gp130 antibodies, increasing amounts of antibody progressively reduced human OSM stimulation of the OSM receptor on HepG2 cells, a reporter gene tested.

Inhibition of Oncostatin M by RNA Aptamers

To determine whether the high affinity aptamers generated in this study were capable of functionally antagonizing the activation of the OSM receptor on HepG2 cells, a reporter gene experiment was performed. The three high affinity binding aptamers, ADR58, ADR120, and ADR152, dose-dependently blocked the ability of human OSM to activate the secreted placental alkaline phosphatase reporter gene in HepG2B6 cells with an IC_{50} of 100 nM (Fig. 2). An oligonucleotide with low affinity, ADR147, showed no activity in this assay (Fig. 3). ADR58 did not appear to bind human OSM at any concentration tested.

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to gp130, a control ELISA experiment was performed. In this experiment, the ability of the aptamer to prevent binding of the primary antibody to assay plates coated with the gp130 receptor was examined. As expected, ADR58 showed no activity in this assay (Fig. 3b), indicating that the effects observed in the OSM/gp130 ELISA are specifically due to the binding of aptamer to human OSM.

A number of experiments were performed to examine the specificity of aptamer ADR58 for human OSM over other homologous proteins. Human and mouse OSM are 42% identical at the protein level. Dissociation binding experiments were performed using human and murine OSM both purchased from R&D Systems. Although ADR58 bound to human OSM with an apparent \(K_d\) of 7 nM, the aptamer did not bind to murine OSM at any concentration tested (Fig. 4). In a second experiment, the binding of ADR58 to another proinflammatory cytokine, TNF-\(\alpha\), was examined by ELISA. ADR58 had no effect upon the binding of TNF-\(\alpha\) to the TNF receptor 1 (Fig. 5). In a third experiment, the effect of ADR58 on LIF- and OSM-induced IL-6 release from KB cells was examined. KB cells are an oral epithelial cell line that releases IL-6 following stimulation with OSM or LIF. LIF is a member of the same gene family and is believed to be structurally related to OSM (47). ADR58 and ADR147 both showed a slight potentiation of LIF-induced IL-6 release from KB cells, which was attributed to a nonspecific effect of RNA (Fig. 6a). ADR58 but not ADR147 showed a dose-dependent inhibition of OSM-induced IL-6 release from KB cells, with an apparent \(IC_{50}\) of 3 nM (Fig. 6b). Finally, the ability of aptamer ADR58 to prevent IL-6 mediated activation of the signal transduction and activator of transcription-secreted placental alkaline phosphatase reporter gene in HepG2 cells was tested. Although ADR58 was capable of dose-dependently inhibiting OSM-induced activation of the reporter gene, neither ADR58 nor the control RNA, ADR147, showed any inhibition of IL-6 mediated activation of the reporter gene (Fig. 7).

The sequence of ADR58 is shown in Fig. 8. By synthesizing progressively shorter oligonucleotides using solid phase synthesis and measuring the affinity for OSM, it was determined that ADR58 RNA could be truncated from 71 bases in length to 33 bases without any loss in affinity for OSM (data not shown). The pyrimidine positions in ADR58 all contain a 2'-fluoro group on the ribose ring. This modification stabilizes the aptamer with respect to ribonuclease activity. To further increase aptamer stability, each individual purine position was sequentially substituted with a 2'-O-methyl purine residue, and the effect of the substitution upon the affinity of the truncated ADR58 for OSM noted. It was determined that 14 of 18 2'-O-methyl purines could be substituted with 2'-O-methyl purine without any loss in affinity for OSM (data not shown). Substitution of the remaining four 2'-hydroxy positions dramatically reduced affinity of the truncated ADR58 for OSM. Truncated ADR58 was terminated at the 3' end with a 3'-thymidine cap to further increase resistance to nuclease attack. The sequence and O-methyl substitution pattern of truncated ADR58 are shown in Fig. 8.

To determine that truncated, modified ADR58 retained functional activity, it was tested in the HepG2 reporter cell assay...
and the OSM-gp130 ELISA. The truncated, modified ADR58 showed activity similar to that of full-length ADR58 in both assays with an apparent IC$_{50}$ of 350 nM in the HEPG2 cell assay (Fig. 9a) and an apparent IC$_{50}$ of 0.3 μM in the OSM-gp130 ELISA (Fig. 9b).

**DISCUSSION**

The elucidation of the role of a protein in both normal physiology and in disease states requires the generation of specific tools that are capable of regulating the activity of that target protein. A number of methods are available to do this. The generation of transgenic animals in which the protein of interest is either overexpressed or deleted from the germ line has provided a powerful tool for the understanding of protein function. However, the generation of a transgenic animal is technically complex and time-consuming, and it can lead to spurious observations due to genetic adaptation. Antisense technology has also been used for this purpose but is complicated by the requirement to ensure cellular delivery of the antisense molecule. The physiological role of many cytokines and other proteins has been determined following the generation of either blocking or activating antibodies to that protein. To complement these technologies, techniques have been developed that allow the generation of specific peptide or nucleotide aptamers to target proteins. In contrast to antibody gen-

**FIG. 6.** Effect of aptamers on LIF- and OSM-induced interleukin 6 release from KB cells. Shown are ADR58 (●) and ADR147 control (▲). Aptamers were preincubated with either 1 ng/ml LIF (a) or 1 ng/ml OSM (b). The mixture was incubated with KB cells for 24 h. Interleukin 6 levels in the cell culture medium were then measured by ELISA. The experiment was carried out in triplicate. Error bars represent S.D. The concentrations of LIF and OSM used were at the approximate ED$_{50}$ for this assay. In the absence of aptamer, the IL-6 release induced by 1 ng/ml LIF was 1113 ± 7.5 pg/ml, and the release induced by OSM was 4702 ± 67 pg/ml. From cells incubated with medium alone, the IL-6 release was 49 ± 9 pg/ml.

**FIG. 7.** Effect of aptamer on IL-6 signaling in the HEPG2 reporter gene cell line. IL-6 signaling via the IL-6 receptor in the HEPG2 reporter cell line results in production of secreted alkaline phosphatase. Shown are ADR58 (●) and ADR147 control (▲). Aptamer and control RNA were preincubated with 4 ng/ml IL-6 (which is approximately the ED$_{50}$ for this assay), and production of secreted alkaline phosphatase was monitored following addition of IL-6/aptamer mixture to the HEPG2 reporter cell line. In the absence of aptamer, cells incubated with IL-6 gave an A of 0.3, and in cells incubated with medium alone, the A was 0.06. ADR58 inhibition of OSM-induced secreted alkaline phosphatase (□) was included as a positive control. The experiment was carried out in triplicate. Error bars represent S.D.
The aptamers generated in this study were 71 nucleotides long and were stabilized by the incorporation of 2′-fluoro pyrimidine nucleotides to protect against ribonuclease degradation (30). Following the generation of ADR58, a number of post-SELEX optimization protocols were performed to increase aptamer stability, to reduce the cost of synthesis, to generate a molecule suitable for chemical synthesis, and to develop formulations for use in vivo. In the first of these procedures, the aptamer was truncated to reduce the length of the molecule to a core sequence required for activity. There are examples in which nucleotides within the fixed sequence have contributed to aptamer affinity (30, 34, 35). In the case of ADR58, a single base from the 3′ fixed region was found to be required for binding OSM. During the SELEX process, RNA is synthesized containing 2′-fluoro-modified pyrimidine nucleotides. It is not possible to include 2′-modified purine nucleotides, as the T7 polymerase used for in vitro transcription will not tolerate this modification under standard transcription conditions. Hence, to increase aptamer stability, it is usual to replace the purine bases within the aptamer with 2′-modified purine bases post-SELEX. This modification is usually through the use of 2′-O-methyl purines. However, this modification may result in a change of affinity of the aptamer. In the case of ADR58, it was possible to substitute 14 of 18 purine positions with 2′-O-methyl purine without any loss in affinity for OSM.

In order to isolate an aptamer that is active under physiological conditions, the selection was carried out at 37 °C and in physiological concentrations of salt. It is highly likely that the presence of divalent cations, such as magnesium and calcium, is required for aptamer binding to OSM. Experiments in this study that have omitted divalent cations have failed (data not shown), and previous evidence (34) has indicated that divalent cations are sometimes involved in aptamer function.

Once an aptamer has been truncated and maximally stabilized, it is possible to make additions to the 5′ end to aid use of the aptamer in vivo. Aptamers have been modified through the addition of both 20,000 and 40,000 Da polyethylene glycol to decrease plasma clearance in vivo (48) or through the incorporation into liposomes through the addition of a diacetylglycerol lipid group to the 5′ end of the aptamer (37). Truncated ADR58 may be further modified for use as a diagnostic molecule to detect the presence of human OSM in serum, tissue, or other ex vivo samples or for the detection of human OSM in whole body in vivo.

### Figure 8

**FIG. 8.** a, nucleotide sequence of aptamer ADR58. The fixed regions are in **boldface**. All pyrimidine positions contain a 2′ fluoro modification, and all purine positions are 2′ hydroxy. b, nucleotide sequence of truncated ADR58. All pyrimidine positions contain a 2′ fluoro modification, and all purine positions are 2′ O-methyl except those marked with an asterisk, which are 2′ hydroxy. The truncated aptamer also contains a 3′-3′ thymidine cap at the 3′ end.

### Figure 9

**FIG. 9.** Functional activity of truncated ADR58. a, effect of truncated ADR58 on OSM signaling via the OSM receptor in the HEPG2 reporter cell line, measured by production of secreted alkaline phosphatase. b, activity of truncated ADR58 in OSM-gp130 ELISA. The ability of truncated ADR58 to block the interaction of microtiter plate-immobilized OSM with gp130 is shown. gp130-IgG-Fc fusion binding was quantitated with an alkaline phosphatase-linked anti-IgG antibody. The experiment was carried out in triplicate. Error bars represent S.D. At the concentration of gp130-IgG-Fc used, the OSM was approximately at the ED50. The A values are corrected for nonspecific binding (where either OSM or gp130-IgG-Fc is excluded; A = 0.06). The A in the presence of OSM and gp130-IgG-Fc but without aptamer was 1.29 ± 0.3.
vivo imaging studies (38, 35). Fluorescein, biotin, or other affinity tags can be added to the 5′ end of the aptamer to aid in detection for applications such as fluorescence-activated cell sorting (38, 40), enzyme-linked oligonucleotide assays (41), and other diagnostic applications. The advent of technetium-99m chelating peptide cages, such as MAG3 (42), has greatly facilitated the use of a wide range of molecules (43) and macromolecules (44) for imaging the presence of the target protein in vivo (45). Recent modification of the technetium-99m chelators has enabled more efficient and stable labeling of molecules under mild conditions (46). Thus, it is foreseeable that the conjugation of a technetium-99m chelating cage to ADR58 would enable the in vivo imaging of OSM in rheumatoid arthritis.

AD58 thus represents a highly potent and selective functional antagonist of human OSM. The aptamers generated in this study may be used to further probe the role of OSM in normal physiology and diseased states such as rheumatoid arthritis. In addition to the use of these aptamers as validation tools, the aptamer in general can also be developed as a diagnostic agent and may be useful as a therapeutic molecule in its own right.

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