S

Industria y Comercio
S U P E R I N T E N D E N C I A

DIRECCIÔN DE NUEVAS CREACIONES

RADICACION EXPEDIENTE No. 6-100210

EL EXTRACTO DE LA PRESENTE SOLICITUD DE PATENTE DE INVENCIÔN

FUE PUBLICADO EN LA GACETA DE PROPIEDAD INDUSTRIAL No 583 DE

FECHA 18 DE DICIEMBRE DE 2007 EL TERMINO HABIL SEIIALADO POR LA

LEY EXPIRA EL 14 DE MARZO DE 2008

NOTA: Dentro del plazo de los seis (6) meses siguientes a.partir de Is fecha de publicación en la gaceta de Propiedad Industrial, deberÆ solicitar y cancelar at examen de patentabilidad so pena de qua la solicitud caiga en abandono, de acuerdo COfl to establecido en at Articulo 44 de (a Decision 486/2000.

SE PRESENTARON OPOSICIONES POR PARTE DE TERCEROS.

SI NO X

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US 6,344,321 131

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Comoglio 193. Las, 65:131-hS: Rubto 1993, Uiochim plaxes to yield a nod-enriched mislaru of Ouclitic acids,
Brophys Ada. 1155:357 -71 Jiawt 1999, Ccii Ecu Oticol then reiterating the steps,E binding. partLionng.diasociat-
IfematoL 29:209-48). The exported leon of c-met is ing and amplifying through as natty cycles as desired to
tinily a pro-peptide which is protelytically cleaved. The yield highly specific high affinity nucleic acid ligands to the
mature protein isa ltetcrodimer consisting of an cxtravellutar target molecule.
50 hUla ci-subunit bound by disulfide bonds to a 1411 kl)a ttha.sbecn_[ecugnar4byat4Jvaasle4hat-a6.
11--subunit. In addition to its cxrtaectlular domain, the SLI.LX nthodamcrstntuu..adrltcattieaijdl
0-subunit has a presemed tr Irane.spanningscqwrncc andsspomtds-can:fcraciwidearmypf$apes,---sims-an
a 435 antittt acid intrucalutar rtc,majn containing a typical conIrguratona,-and-aae-capahte-ol-a-taebcpemirtf
tyrosne Idnase.
is produced primarily by oresencitymal cells, while
c-mel is mainly expressed on cc-lls4pithlil origin. I[GF flisbasic SULI.iX method haste-ea modified to achieve a
is very highly conserved at the amino acid levi between number of specific objectives. For example. U.S. patent
species. This homology extends into the functional realm as application Ser. No. 07196(U193, filed Oct. 14, 1992, now
observed in mitogetile stimulation of hepatocytas in culture 5 abandoned, and US. Pat. No. 5,707,796, bath entitled
by LIGF across species, including human, rat, mouse, pip "Method for Selecting Nucleic Acids on the Basis of
and di& ills indicates that human HUE can be used Structure," describe tire use of (he SELEX process in coo-
cam-specifically in a variety of assays. junction with gel ekietrophuresis to select tacleic acid
Given the roles ofHGF and c-tutet in disease, it would be molecules with specific structural characteristics, such as
desirable to have agents that hind to and inhibit the activity beat DNA. U.S. patent application Set. No. 08/I 23.135, fated
of these proteins. It would also be tirsirtibte to have agents Sep. 17, 1993, entitled "Pluttoselection of Nucleic Acid
that can quantitate the levels of Il(,F and c-met in individual Ligauds,", now abandoned. U.S. Pat. No. 5,763,177eulided
in order to gather diagrtanilie and prognostic information. "Systematic Evolution of Ligatads by Exponential Enrich-
dogma for many years was that nucleic acids had meet: l’horoscLeetion of Macide Acid ligaatds and Solution
primarily an informational role.Througha method koownas 75 SEI.ItX" and U.S. patent application Sri. Ni. 094193,293,
Systematic Evolution of Ligrands by EXponential Ilteti ten. 8, 19911, encitld "Systematac Evohition of ligands
enrichment, turned the Slit 1iX process, it baa, become clear by Exponential llnrichment: Photnselaction of Nucleic Acid
that nucleic acids have three dimensional structural diversity Ligattds and Solution SELEX" describe a SELEX based
not unlike proteiesheSELEXpgcussia.methodiot.the method for selecting nucleic acid ligands containing photo-
rcacaive groups eapabteof binding and/or photocrosslinkictg
iutcitindingitoiaagetmotecnldaind1sttesccibethicttL& to and/or plintOinactivating a target molecule, US. Pat. No.
5,580,737 entitled "lligh.Al3oity Nucleic Acid Uganda
eesidsdSystemaiic-EvointtCliiliii?al Thai Discriminate Between Theophyllinc and Caffeine."
Enrichment," now abandoned, U.S. Pat. No. 5,475,t196 describes a method for identifying highly specific nucleic
entitled "Nucleic Acid t.igantls, US. Pat. No. 5.270,163 acid ligands able to discriminate between closely related
(sec also WO 91/19813) entitled "Methods For Identifying tnctlecuiles, which can he non-peptidic, termed Counter-
NuIcic Acid Ligatids" each of which is spccilically incur- StilEX. US.. Baa-No. 55,5fi7,588 entitled "Systematic Lvii-
pecrated by reftrence herein. Each of these applications, tutinn of Ligands by EXponential tincidmnteatr: &abution
collectively referred to herein as the SELEX Patent Si1.LX/’daacaibcsa SEI.ldX-bused method which achieves
A.iplkatioaa describes a fitodantentally novel fliutlmd for ii highly efficient partitioning between oligonuctceitides has.
making a nucleic acid ligated to any desired target ing high and low affinity for a target molecule.
The SELEX process provides a class of products which are ThuSliL’-methrsi-wnnompastbe4dentiflcrttirinitf
referred to as. nucleic acid ligands orraptanjets, each havingjaaijltraftuLyJauturltricdagjatrdihad.nudei
a unique sequence, and which has the property binding ç6’ideSc4nibrtirtittPSticbarnetedStkSou_thrt.hgatkd
-specifically to a drained target counpoutad or nolecule.Each..st clrasiSedhgetyriropecved-detivrry

iifilLihilitilEa4iaritor phosphate
SEE,IiX prrsess-idtntilictE9iudliti

1iwttetherinonarcric-rtr7toT awricrthdEniules of eeitytmo4itiud-at thai S-aixi 2 -posit its of primidiaaes. U.S
targets. The SELEX
method applied to the applktion of high slImiLy.ln’uudiitg ay’aedr1igedseontainrmore.rttacleotides.rnodi1
involves selection from a mixture of candidate eatinanacle- ’4tkni..42-fiueru:lndnc-2iEtrrnet1aylJ
otadcr, and step-wise ttcrrattoas of binding, partitioning and (2 -GM azentapphattmvSert’uo-ttisii641l24 ftlerL
amplification ca.stng the seine general selection selienie to lttfl22,..l4entdIctL..Nuaie1..NIdbtsLiifJ&epar4Uofl..t4i
achieve virtually any desired criterion ofhanding affinity citI
selectivity. Starting from a mixture of nucleic acids, prefer- at Ii ckuphilidi)tyfl2iicirw rdiiilitmit,al. ala ahauc ably comprising a srgmett of randomized sequence the
Slit.1IX method includes steps of contacting the mixture
with the target eider conditions, favorable for binding, The StililiX method encompasses combining selected
partmttotam5, unbound nucleic acid. From those mictntc &ctdit olrgomtcleotadns with oiler selected oltgonuckcat&s and
which llavc hound specifically to target rrauleccths, dissoci- 65 ncn-saligonttcleotidc functional units as &lcserabcd 16 U.S.
ating that nucleic acid-target cottiplexe,s, amplifying the Pat. Nrc 5,637,459 entitled "Systematic Evolution of
rtudcua, acids dttsocatk4 fnamaa Ills tc ic. anal-target ion I-igards by IaXp ucntial linriebmulL Cbtataeri. SEt Lx

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and U.S. Pat. No. 5,683,867 entitled "SystiWatic Evolution FIG. SB shows conventional poo1 binding.
of Ligaodsby EXponenalal Enrichmeut Blended SIILEX." 116. 6(A.14) illustrates inhibition of long/mt 11(11 stimu-
respectively. These applications allow the combination of latioun of starved IIUVE(’s by aptamem

the broad array of shapes and other properties, and the trif;. 6A shows a lid sri of aptanitrs. 110. 60 illustrates efficient amplification and replication properties, of oligo- 5 ii 2nd set of aptarncrs. nucicotides with the- e1rsirahleproperlieofotfa,-r molecules F’lU 7A,l1) illustrates truncates of aptamer 8-102 11(1.
7A shows predicted two-dimensional structures of intl. 5Cl0.tfiidhdtt’i!i!i]II length and tnrttcated 1W 714 %hows binding of

full-length and truncated aptamers up Iii I in e I-If 8(AB) illustrates truncates 01 aptatnter 8-17 P16 patent application Sec. No. 08/434,465, flied May 4, 19951 RA shows a predicted two-dimensional structures of full- emitted "Nucleic Acid Ligand Complexes". Each of the length and truncated seupacnecs. FIG. 811 shows binding of above described patent applications which describe modtft- lull-length and truncated aptamers to 1101 cations of the basic SIi1.EX procedure are specifically

incorporated by referance herein in their entirety. Elf 9(A,lfl illustrates binding of 1161 truncate SIIIEX
It is an object of the present invention It obtain nucleic pools. 110, 9Ashows the 1-IGF SELEX 430197 series FIG.

9filshows. the IlfJF SEIJiX S 30N7 series acid ligands to 1161 and c-met using the SLIEX process. PIG. 10 ShOWS aptamer inhibition of 100 np/mi IMF It is a furtber object of the invention to obtain nucleic acid stimulation of 4MBr-5 cells. ligands that act as inhibitors of HOP and c-met. 20 FIG. L1A,B) illustrates aptamer inhibition of SO np/nil It is a further object ofthe invention to provide therapeutic ILOF stimulation of 4MBr5 cells. and diagnostic agents for lumorigcntc conditions an which 1-16. 1 1r4, shows the effect of PEGylalion of 36tner. 110. HOF and c-met are implicated. IIB shows a comparison of PEGylated 36mner to brat It is yet a further object of the invention to use nucleic acid full-length inhibitor 8-17. ligands to IIGF and c-met to diagnose and treat at FIG. 12 shows aptamer inhibition of 50 rig/tail TIGIt hypertension, arteriosclerosis, myocardial inlllrcluori, and stimulation of 4MBr-5 nails. rheumatoid arthritis FIG. 13 shows IfIiVEC witogenesis by 10 up/mi 1IOF, 10 his an even further object of the invention in use nucleic imp/mi VECF. cr both hOP and VEGF. acid ligands to HGF singly or in combination with other 116. 14(A-C) illatstmates aptamer-mediated inhibition of nucleic acid Ligands that inhibit VEGF and/or bFOV anctitar ia HCVEC ntilogenesis. FIG. 14A shows stimulation by both possibly other arigiogenesas factors. 110F and S/LOP inhibited by either FLOP or VEGF aplamaters

SUMMARY OF ThE INVENTION er both.
PIG 14B illustrates stimulation by ITOF alone inhibited
Metlttmmts are provided for generating nucleic acid ligands as by either ItOh" or VEGF aptamer or both. FIG. 14C illus-
to 1101 atud c-met. The methods use the SE1.EIX process for tratcrastimulation by S/LOP alone inhibited by either 1101 or
ligand gmtnaaratiott. The nucleic acid ligands provided by the VEGF aptamer or both.

invention we useful as therapeutic and diagnostic agents for 11;. 15 depicts ratios of selected to nnmemtccted partially a number of diseases. 2 -0-methyl substituted purints in aptamer MX22354.
- . at BRIEF DIiSCRWUON 03 FIIIi DRAWINGS

FIG. 16 illustrates 2-0-methyl substituted derivatives of
19X22354 binding to ILUFt average of two experiments.
FIG. I illustrates the template and primer ollgonucle 1-10. 17(A-(’) illustrates binding of SiI,liX pools to
otidass ti.saul 2’.tt:pyrimidlne RNA SELEX experiments. ’lie C-met. FIG. 17A shows c-Met SEI.L7c 40N7. HE, 1711
5’ lixed region of the template and primers contains a 17 shows c-Mel SELEX 30N8. FIG. 17C shows both SEL-
promoter to facilitatti transcription of RNA by TI RNA 45 fiXes: a. c pools, 40N7; hi, d pools. 30N8.
polytaterase. FIG- is illustrates binding of c-mci SELEX pools tti
FIG. 2 illustrates RNasclt cleavage primers used in c-tact and KEEL Ig fusion proteins.
hybridization truncate SELEX. Bases depicted in hold-type PIG. 19(A.B) shows binding of c-met 40N7 cloned
are 2’-0-unetltyl modified and bases underlined are daioxyri- aptaoaaira to at-met and Kl)R Ig fusion proteins. FIG. EtA
bonucleosidais. The rtutdttni region is designated as "N". 50 shows clone 7c.1. FIG. 19B shows ckne7c-3.

1/pout treatment with biNasell, the fixed regions are removed UltflikItiiD IJESCIWa’IlON OF tilE at the positions indicated by the carols. Note that the there PI1IIFEIIREJ) EMBODIMENTS am two possible cleavage sites at the 5-prime end of the

fixed region, resulting in RNAwhich has one or two fixed the erurial method utilized herein for identifying nucleic
U vstduas. as acid ligands to (lGlatad c-met iscralled the SELEX pros’arss,.
-

3(A,31) illustrates handing of SELEX peals to 11(1-. an acronym for Systematic Evolution 01’ Uganda by Expo-
FIG. 3A dews HOP SELEX t 30197 pools. FIG. 319 shows nential enrichoanart and involves (a) contacting the candidate

rnixaarr,r of nucleic acids with IIGF or c-met, or expressed lIOF SEE.EX 2 30N8 Pools, dtmaaimii or peptides corresponding to 11(11 or c-met, (Ii) 110. 4(A,11) illustrates two methods of evaluating 1101" an members ofsaid candidate mixture on 511ELEX 3 30N7 pool binding to 1101. in FIG. 4k, heparin partitioniitgtaetweaao
the ISs.SiS of affinity to 1101 or c-met, and c) amplifying the. competes with RNA pools for binding to 2.7 nM I tOE trIG, selected molecules to yield a mixture of nucleic acids 411 illustrates conventional pool binding. emtrictiml for nucleic acid sequences with a relatively higher ItO. S(A,H) ilIstrrmias two methods of evaluating IIGF affinity for binding to IIGP 0cc-met.

SELEX 3 30197 pool hinting to 110F. as tIetiritions
1/16’. SA shows that iRNA competes with RNA pools for Various tents are used herein to rider in aspects of (lie
binding to 2.1 nM [lOP present invention. To aid in liii darifacralic’mt 5af the ttescrp-

lR1llhl!IllIHIUS(X)6344.121 III

(12) United States Patent (111) Patent No.: US 6,344,321 B!
Rabin et at. i’ts Date of Patent: -Feb. 5,2002

(54) 1N13WC ACM) CAN 8 Wt*fWWND TO 5,731,424 A 3t1698 Tootitnuin at at..........536/23.1
NpAT(xroH:JwCTotscrrl1w--... 5,134,1134 A 3/1098 Jayaaena a al............ 536/23.1
ACTOR(acF/S)oRrrS4181e81pro-- 5,1137,834 A 11/1998 Pagiath eL at.............516/23.1
FoIustGN PATENT DOCUMENTS

(75) Invuitors: Ross Rabin, faycttn Michael 00 2 183 661 A 611987
Locbrle, Louisville; Ncbojsn Janjie; WO W089/18694 1119119
Lame Cold, both of Jloulthr, all of CO Wo WO 9VI9813 12/091
(US) Wo %V0QZf14843 911992

WO W004/66909 3/1994

(73) Assignee: 011usd Sciences, Inc., Rater City, CA
(US)

() Notice. Subject to any disclaimer, the term ofthis
patent is cittetided or adjusted under 35
U.S.C. 154) by 0 days.

This patent is subject to a terminal 6/a-
clainier.

(25) AppL No.: 09/364339

(22) Filed: Jul. 211, 1999

Related V.S.Application Data

(63) Coolinentien.ia-pait of application No.1)9/502,344, flIed on Aug. 27, 1098, whith is a,a,ntiuuatjoa at application No.

LBM6P,605, filed on $uu. 6, 9)95. sow Pat. Ne. 5,843,653. whirl, inn eoathmtfon otepphcalion No. 07/214,151, filed on Jun. 10, 1901, now Par. No. 5.475,1196, which is a nuufljj.pa ufapplication No. 07/536,428, iSle) on Jun. 10. 1081), now abandoned.

(51) laiL Q.’........................COlD 21/04; C07H X02;
(’124.) 1/68; C12P 59134
(52) U.S. CL.........................435/6; 435/911; 536/23.1;
536/24.3

(514) Field or Search...................435/6, 91.1; 536j23.1,
536/243

(56) References Cited

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OThER PU811CAIIONS

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(List continued on next page.)

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L.LC.

(57) ABSTRACT

The invention provides nucleic acid tigands to itepatocyte
growth factotitcatter factor (I-(G(7) and its receptor c-tact.
The nucleic acid ligainds ofthe instant invention ale isolated
using the SELIIX method. SELEX is an acronym for Sys-
tematic Evolution of iigands by (iXpotrential enrichment.
The nucleic acid iiganiis of the invention are useful as
diagnostic and therapeutic agents for diseases in which
elevated 110(7 and c-mel activity arc causative factors.

3 Claims, 23 Drawing Sheets

US 6,344,321 LII

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NUCLEIC ACID UGANUS 91111CR DINt) TO Elevated Levels of DO? and c-tent have also been
H14.PATOCYTE GRowfll?ACTORlSCATF1R observed in rnoo.oaeological settings, suck as 4ypertenskan
FACTOR ltJS1) OR ITS njv (Morishits, Aoki ci at. 1997.1 Athetoatter Throirab. 4:12-9;
C-MST Nakamura, Motigitcbi et at. 1998, Blacken. Diaphys ltcs
Common. 242238-43). arteriosclerosis (Ninhimuaa, UsIa-
CROSS RIIFERIINCETO REIATEI) lyama et al. 1997,.1 t-Iypertens. 15:1137-42; Morinthita,
A3’PLJCMIONS Nakamura Cl al. 19911,1 Atheroscler Throtub. 4:128-34),

This application is a continuation-in-part of U.S. patent myocardial infarction (Sato, Yonhinouchi et at. 1998,.1
application Set. No. (19/502,344, filed Aug. 27, 1998, CudioL 32.77-.82), and rheumatoid arthritis (Koch, linda-

ram ci at. 1996, Arthritis Rheum. 39:1565.-is), raising entitled "Nucleic Acid uganda," which is continuation of In possibility of additional therapeutic and diagnostic applies- US. patent application Sin. No. W469.1609, filed Jun. 6, floss. 1995, entitled "Method for Detecting a ’target Molecule in The mole of FIGI1/c-nimet in metastasis has been elucidated a Sample Using a Nucleic Acid Ligaxid." now U.S. Pat, No. in mice using cell tines transformed with lIGF/c-nant 5,843,653, which isa continuation ofU.S. patent application (reviewed in reference Jeffers, long ci at. 1996,3 Mid Med.

Set.No. (17/714,331, filed Suet. 10, 1991, entitled "Nucleic 25 74505.-33) In another metastasis model, lantauan breast
Acid Ligands," now U.S. Pal. N. 5,475,096, which ts a carcinoma cells expressing HCfl7/c-ntet were injected in the

continuation-in-part of U.S. patent application Set. No. mouse mammary fat pad, resulting in eventual lung 417/536,428, filed Inn. 10, 1990, now abandoned. memastases in addition to the primary tumor(Meiners,Iltink-

f’IELI) OF THE INVENTION mane t aL 1998, Onnogene. 16:9-20). Also, Irausgertic 25 mice which os’erexpress HG? become tumor-laden at many This invention is directed towards obtaining nucleic acid loin (Takayatna, LaRoelaulle ci id. 1997, Proc Nail Aced Sci

ligandsofkcpatcicyte growth factordanitar factor(IloP) and U S A. 94:701-6).

sreteptcarc-anet. The method used in the invention iscatted None of the data mentioned above provide proof of a SHLSX, which man acronym for Systematic Evolution of direct causative role of HGF/c-met in human cancer.
Ligands by lhXpottential enrichment. The invention is also 25 although the accumulated weight ofthe correlative data are
directed towards therapeutic and diagnostic magenta for convincing. However, a causal connection was established
diseases in which elevated 110(7 said c-mat activity are between ge/rn-line c-met mutations, which constitutively
causative factors. activate its tyroslun kmnase domain, and the occurrence of

lonnami papillary renal carcinoma (Schmidt, Dub ci at. tOP?, BACKGROUND OF THE INVENTION 30 Nat Gent. 16:68-73).
Iteparocyte growth factor/scatter factor (abbreviated Recent work on the relationship between inhibition of
herein as 11(23") is a potent cytoltine whivb, through inter- angiogenesis and the suppression or reversion of tumor
action with its receptor c-met, stimulates Proliferation, progression shows great promise in the laeaanieot of cancer
morphtrganesis, and migration of a wide variety of cell (Boehm, Fotkmama et al. 1997, Nature. 3911:4114-7). In this
types, predominantly epitbalial HG? and c-met ace involved 35 report, it was shown that the use of multiple arigiagenesis
in several cellular processes involved in itimninigenesia, Inhibitors confers superior tumor sappressionfregression
notably angitagenesis and motogenesis, the tatter having compared to the effect of a single inhibitor. Angiogenesis is
been implicated in the migration of cells required for markedly stimulated by HG?, as well as vascular cridothelini
mntas1zsls (reviewed in references Jiang and Discos 1997, growth factor (VISe,?) and basic fibroblast growth factor
Itistid lilstopathot. 12:537-55; Tamagnonre and Qimmnoglio 4o (b?Gt) (Rosen, Larnszus ci at. 1997. Ciba Found Symp.
tOP’?,Cylokinc Growth Factor Rev. 8:129-42;Jiang, Iliscoxe 212:2(5-26). HG? and VEG? were recently reported to
et al. 1999, Cril Rev Oncol llenaaeol. 29:209.-48). have an additive or synergistic effect on mitogearsis of
Interestingly, proteases that degrade the extracellular matrix larasran umbilical vein endothelial cells ((IUVECs) (Van
also activate 110?, which in turn up-regulates umkina.se type Bette, Wilxemnbichter em at. 1998, Circulation. 97:381-90).
plasminogen activator(liPA) and its receptor, insulting in an 4j Similar combined effects are likely to contribute to anglo-
activating loop feeding the invasive said migratory processes genesis and metastasis.
required for metastatic cancer. Human lIGtt protein is expressed asa single peptide chain
HO? and the c-met receptor are expressed at abnormally of 728 amino acids (reviewed in references Mtcnauo and
high levels in a large variety ofsolid tumors, in addition to Nakamura 1993. inst. 65:1-29; Ruble, llcnitaro ci at. 1993,
numerous demonstrations in vitro of the effects of UGlYc- sr Binchina Biophys Ada. 1155:357-71; hang 1999. Cdt Rev
noel on the behavior of tumor colt lines, the levels of 110(7 Omrcot 1tcniatol. 29:2(19-48). The amino-terminal St residue
and/or c-met have been measured in tanimman tumor tissues signal sequence of HG? cleaved upon export, followed by
(reviewed in reference Slang 1999, Cdt 11ev Outsit Hemnatal. pnoteulytic cleavage by uPA arid/or oilier protease’. The
29.209-48). 111gb levels of HG? and/or c.nuct have been mature protein is a tieterodiuner consisting of a 463 residue
observed in liver, breast, pancreas, lung, kinluoy, bladder, as cessbunut and a 234 residue Il-subunit, linked visa single
ovary, brain, prostate, gallbladder and myelotea tumors in disutlldms bond. RG(7 is homologous In plasminogen: its
artttieinn to many others, a-subunit contains an N-terminal plasminogeit-activatec-
For several of the cancer types listed above, the ptogsos- peptide (PAP) followed by four kringte domains, and the
tic value of omensnrirtg 1101-/c-met level, has been evaluated 9.subuanil is a aerimte prarea.se-lilre domain, inactive because
and found no be potentially itsefimt for determining the en it lacks critical catalytic amino acids. The recently solved
progmaucion and severity of disease. The correlative data are crystalstructure of an DC.? fragment containing PAParnel the
strongest in the case of breast cancer (Ghou.ssoub. Dillon et first ltrirmgtc domain indicate that this region is responsible
aL 1008. Cancer. 82:1513-211; Toi, Tanignetni el al. 1998. for heparlo intending and dinacrization (Ctnirgadre, ttepple em
(’tin Cancer Res. 4:1159-64), and non-small cell lung cancer aL 1999, Nut Strad Bin). 6:72-9), in addition to receptor
(Siegfried. Weissleld ci at. 1997, Cancer Res. 57:433-9; at interaction.
Siegfried. Weissleld of at. 1998, Arm ’(’licarac Smirg. llumtan c-mot protein is exported to the cell surface via a
66:1915-14). 23 amino avid signal sequence (mvicwetl in references

US 6.344,321 131

it

(igand can be made to wcniaac the in vivo stability of the
mickric acid ligand or to enhance or to mediate the delivery
oldie nucleic acid ligand. See, e.g., U.S. patent application
Set- No. 081117,qd I, tiled Sep. 5, 1993. now abandoned. and
U.S. Pal. No. 5.660,985, both entitled "high Ahhinily Nucleic
Acid l.ganthr Containing Modified Nuelideni", and the
U.S. Patent Application entitled Trcciptk,n-free
SELEX", US. patent application Set. No. (192,575 filed
Jul. 28, tUl’J, each of which is specifically incorporated
herein by refenoce. Modifications of the nucleic acid
ligands contemplated in this invention include, but are not 10
limited to, thriac which provide either chemical groups that
ruccirpocatr. additional charge, polarit’ititb.
hydrophobicity. hydrogen bonding, electrostatic interaction,
and Iluxiounhity iit the nucleic acid ligand bases or to the
nucleic acid ligand as a whole. Such modifications include.
but are tart limited to, 2’-position sugar modifications.
5-position pyriruidinc modifications. 8-position purinc
moditications, modifications at exocyclic minions, substitu-
tion of 4-0tienuriditte, substitution of S-hromo or S-lode-
uracit backbone modifications, phersphorothioale or alkyl 20
phosphate nnorlillcatimns, mcthylaiions, unusual base-
pairing combinations such as the isrihases isecytidine nod
isciguanidine and the like. Modlticalkins can aLto include 3’
and 5’ modifications such as capping. In preferred embodi-
ments of the instant invention, the nucleic acid ligands are
RNA etolecules that are 2’-fluoru (2’-F) modified on the
sugar moiety of pyritnidinc residues.

the modifications can be pet- or post-SEUiX process modifications. Pm,-SELEX process modifications yield
oucteic acid ligainde with both spcciftcity for thaw SELEX
target and improved in viva stability. Post-SELEX process
modifications made to 2’-OH nucleic acid ligands can result
in improved in vivo stabilitywithout adversely affecting the
binding capacity ofthe nucleic acid ligand.
9tltet modifications, are known to erie ofordioaar skill itt
the art Such modifications may be made pcatt-SLLUX
process (niodilicnrtin of previously identified uannodulled.
ligands) or by incorporation into the SELEX
The nucleic acid ligainds of the invention are prepared
through the SELIiX methodology thai is outlined above and
thoroughly enabled in the SIILEX applications incorporated
herein by ntferrahcr in theiretirirety. The SELEX procraiscan
be performed using purified liGt0or c-met, or fragments
thereof as a target Alternatively, full-length tI()I? or c-met,
or discrete tlonarriits of ITGF or c-met, can be produced it, a 45
suitable expression system. Alternatively, the 5111 EX pro-
miss can be piteforitted using as a target a sytttltctic peptidu
that includes sequences found in Dliii or c-met. I)eternni-
nation of the precise number of amino acids needed frthe
optimal nucleic acid ligand is marine experimentation for
skifledartinims.

to seine crubotlinrerrts, the nacleic acid ligands become
covakualiy attached to their targets upon irradiation of the
muddle acid ligand with light having a selected wavelemngtla.

tl4ctbcids for obtaining such nucleic acid ligands are detailed so in U.S. patent applicatiota Set. No. (161123,93S, tiled Sep. 17.
1993. entitled "Photcr*nelectioo of Nucleic Acid Ligarids,’,
now abandoned, U.S. Pat. No. 5,70,177 entitled "System-
atic Evolution oft.igaaidx by Exponential linricluneant: Piras-

toseteclion Nucleic Acid Ligannis and S.olration 5111 EX" to and U.S. patent application 5cr. No. M/093,293, filed Jun. 8,
1999. entitled "Systtnntativ Evolution of Ligands by Ejipo-
mnential Enrichment: Pltotnselectkmn of NueleicAcid l.irtandnt
and Solution SELEX" cacti of which is spucihicaily incor-
porated herein by refrirritmee in its entirety, a,
In prnrfcrred the Slit paunso is carried
out using (UhF or c-met attached in a solid support. A

12

candidate inaixtme ofsingle stranded RNA irnolenarlcri is then
contacted with the solid support. In especially ptofertatd
ranntnoetimnats, the single stranded RNA nrolucuim have a
2’-lhtnort, modilicalion on C and U residues, rather than a
2’-Otl group. After incubation for a predetermined time at a
selected temperature, the solid support is washed to remove
unbound candidate nucleic acid ligand. ’line nucleic acid
ligands that bitd to the flOP or c-met protein are linen
retersect into solution, [hen reverse transcribed by reverse
tramarcriptssc and amplified using the Polynurrase Chain
Reaction. The amplified candidate mixture is than used to
begin the neat rotmtj of the SELEX process.
In the above embodiments, time solid support can tat a
nitrocellulose fitter. Nucleic acids in the candidate mixture
that tin net interact with the immobilized 11(11 or c-runt can
he ruomovuil from this nitrocellulose titter by application of
a vacuum. in rather tnboditnrnnts, the HOP or c-met target is
irtisotbud on a dry nitrocellulose filter, and nucleic acids in
The candidate mixture that tin not bind to the tflili or c-met
are removed by washing in buffer. In otlserennhodinaents. the
solid support is a microaitrnr plate comprised of. for enrnttnpba,
polystyrene.
In still other embodiments, the ODin or c-met protein is
used as a target for Truncate SELEX, described in U.S.
patent application Scar No. 09/275,850. IlIeci Mar. 24, [999,
entitled -The Tnmcatinta SELEX Method", incorporated
herein by reference in its entirety.
In pnitfr.rrcrl embodiments, the rinirdeic acid ligatnds dirts
obtained attn assayed for their ability to inhibit the uGh/c.
met interaction. In one embodiment, this is performed by
peribrminga citli migration assay. Certain cell types,such as
A549 lung carcinoma cells, wilt show increased migration
through a Matrigitl-eoaled filler insert (Becton l)iekiiioti) in
time presence of(loft.Thus, the degree of inhibition ofUGF
activity in the presence of an PuP or c-met nucleic acid
ligand can be assayed by determining camber of cchic
that have migrated through the filter in the, presenceot’IIGE

H. Methods and Compositions For Using Nucleic
Acid Ligancis In Tteat and Diagnose Disease

Given that elevated levels of c-met and IHIF are observed
in hypertension, arteriosclerosis, myocardial infarction, and
rheumatoid arthritis, nucleic acid tigandswiltserve as useful
therapeutic and diagnostic agents for these diseases. In same
embodiments, inhibitory nucleic acid tigantis of ItriF and
c-met are anintinistered, along with a pharmaceutically
aaaqrted exeipient to an individual suffering from one of
these diseases. Modifications of these nucleic acid ligands
are made in some embodiments to impart itierea.sed stability
upon the nuelcic acid ligands: in the presence of bodily
lintitis. Such modifications ace described and enabled in the
Slit.tnX apphicritions cited above.
In other embodiments, nucleic acid higainds to ilCiF and
c-met arc used to measure the levels of these proteins in an
individual in order In obtain prognostic and diagnostic
irtfoamation. Elevated levels of c-met and [lOP attn asstwi-
auxl with rinriots its the Ever, breast, pancreas, lnutg, kidney,
bladder, ovary, brain, prostrate, and gallhtaafrimnr. Elevated
levels of IIOF and 52-met arc also associated with myvlc’naa.
tn other embodirnenl.ir.nuetcie acid ligands that inhibit the
(lGFIc-rrxet interaction are itmnd to inhibieturtocigecnianis, by
inhibiting, for exantplc,ittigiogetiesis and irtotogenesis.
t,roricrnmnbodiment oldie instant invention, a nucleic acid
Iigaud to HOP is used in combination with nucleic acid.
ligandri to VhlGir(vascular radotloetial growth factor) and/or
bFOF (basic fibtUhilart growth factor) to inhibit tumor

US 6,344,321 [B

13 .rurtastasi.s and artgiergeunrnsis. Thin use of multiply nucleic

acid ligands is likely to have an additive orsynergistic effect
on etintor suppression. Nucleic acid ligands that inhibit
ViiGt are described in U.S. Pint. Pins, 5,849,479, 5,1111,533,
and U.S. patent application 5cr. No. 119/I 56,824, tiled Sep. 3
IS, 1998, each of which is entitled "High AfihmiIy Oligo-
utncleotide tigioads to Vascular titndotkeliah Growth Factor",

and each of which spccifically incrrzpornmttnd herein by
reference in its entirety. Nucleic acid ligantds to VEGF are
also described in (iS, [’at. No. 5,959,228, U.S. patrol 10
application Set. No. (ev5713,930, filed ItO). ft. 1997, U.S.
patetntapplicatiou Sec No. 09/897,351, filed Jul. 21, [997,
and U.S. patent application Set. No. 09j254,ttfiS, filed Mar.
16 1999, each of which is rinitilled "Vascular Enickrthctial
Growth Factors (VEt3l.’) Nucleic Acid Ligatnrl Complexes, is
acid each of which is specifically incorporated by reference
in its entirety. Nucleic acid higands to hFGt" are described in
U.S. Pat. No. 5,639,1168 entitled "High Affinity RNA ligands
for Basic iibrcblast Growth Pacror", and U.S. patent appli-
cation Ser. No. 08/442,423. Meet May 16, 1995, entitled 20
"High Alhinity RNA Ligatacts for Basic Fibroblast Growth
Factor", each of which issp.xnihically incorporated herein by
reference in its entirety.

EXAMPLES as

The following examples are given by way of illustration
only. They rite riot to be taken as limiting the scope of the
invention in any Way.

Materials and Methods

In the sectious below entitled "Results: HQF" arid
"Results: c.mrict"a the following materiaLs and methods were
used:
?totcins 35

The hUh protein and c-meo-tg(2,-His5 fusion protein,
which were tried or the SELEX procrns.s and the EDit-EgG,-
Hhsp proteins were purchased from R&D Systems, Inc.
(Minneapolis, Mint,.). The human c-met-IA-His,, fusion
protein--described From the amino to the earisnatyl to
tertninus--vonnrists of 932 amino acids from the extracellu-
lar domains of the a and (I chains of c-met, a factor Xa
cleavage site, 231 amino acids from human lg(i. (Fe
domain), and a (lis)5 rag. ’Ibis protein is referred let in the
text and figures ante-met Asitniirtr fusion [Irotinjit nnuntnunurtg 45
the vnsittrtar easlethelial gronsrili factor receptor ((1)11 will be
referred to as EDIt.

Anti-tNW nnoncIomnsl antibody MA11294 was purchased
from fiAl) Systems, Inc. Slmnan tgCi was produced
in-house by stable expression from Chinese barrister ovary to
cells.

SlitLX ibmpfates and Primers
Standard SE!AiX templates carrying 30 or 441 random
tntclecnlklc.s flanked by fitted regions of the N7 or 315 series
and associated printers (PIG. I) were used as descrIbed 55
(Fitzwater and Polisky 1996. Methods Entymol.
267:275-301). Truncate SELEX was done by the hybrid-
ization method described in U S. patent application Set. No.
(199275,850, tiled Mar. 24 1999, entitled "’The ’I’runcation
SISLEX Method", incorporated herein by reference in its me
entirety, using t&NaseLl cleavage primers (tilt. 2)
SEII-N Methods

Initial HGF.519 LX experiments were clone by two.
closely-related partitioning methorts, both involving sepa-
rating free from bound RNA on nitrcntreilakr.se litters. Cr111- 55
ventional SLIUiX involves mixing target protein and RNA
lihctty it, l{IISMC hrtffet (lrtnpvs-t,uffcred saline; 25 mM

14

trepes, 137 ntM NaCl. 5m KO pints I toM Cat/i0 1 mM
MgCl p11 7.4), followed by filtration on nittotndllolesc
under vacuum. Maintaining vararirny. the tiller is washed in
lnnfitr, followed by vacuum release and RNA eactenetita. In
spot titter 511 fiX, the protein Is applied to a city nattmocel-
Inalrusu 13 mm tiller, allowed to ad.srnnb for several,ninutce,
then pre-incrrharcd in Huller,’; (IIlhSMChulyer plum 0.1121,
each of Ilcoll, polyvioylpyrmtidorit, and human serrinrn
athurnin) for 10 minutes at 37’ C. to remove unbound
Protein. The wash buffer is nmtttoved, and then the RNA
library is added in the some hmtlTinr. and incubated with the
prutrnin4aouttd filter. The filters are washed by repeated
hrcubatkmn.s in flush buffer. followed by RNA extraction.
SEL.IiX was initiated with between I and S utttoies of
2’-fluoru-pyrinaidice RNA sequence libraries containing
either a 30 or 40 nucltutidc raraekrmiaed region sequence
(11th. 1). The RNA libraries were transcribed front the
ccirtetrpondintg synthetic DNA templates that were generated
lay Ktenrsw, extension (Sambrook, Prir.seh el al. 1989,
3:11.12). The DNA templates were transcribed in I nil
reactions, each containing 0.25 nM template. 038 pM 17
RNA polytneranse. 1 mM each of/SW and 0Th 5 mM each
of 21_In_CTt’ and 2’_ti.UTht, 40 mM ’this-lIt/i (4114 8.0), 12
mM MgO, 1 moM.cpernridine. 5 mM l)’l’L (1.00217, Triton
X-htlO and 4% polyethylene glycol (wfv) for at least 4 hours
at 37° C. The full-length transcription products were purified
by denaturing pulyacrylamide gel etectrophcnresis. Radiola-
beicd RNA was obtained from transcription reactions as
described above, but containing 0.2 mM /SFt5 and I(JJpCi of
30p/Sl? Alternatively, radicnlabated RNA was obtained
by labeling the ti’.end of RNA with ZneP.MP (NEN.
DuPont), ralalyzot! by T4 polynueleotide irinase (New
England Riolabs). To prepare RNAeoutaitting 5-0H groups
for kittase ranacliomns, transcription maclinus included 5 mM
gtiaitoLsiflc.
For conventional tiller SELEX, radiolabeled RNA pools
were suspended in RIISMC buffer to which HOP protr.’itt
was added, and incubated ai 37" C for 30 minutes to hours
depending ott the round. Binding teaciie,ttrcwexe then filtered
under suction through 11.45 pm nitrocellulose filters
(Millipore), peewct with bmndintg heifer. The filters were
immediately washed with nil least 5 ml of HRSMC buffer,
For each binding reaction, a protein-minus control reaction
was done in parallel in order to det52trtmimnsn Itte ananrrnt of
background hireling to the filters. The amount of RNA
retained on the filters was quantified by Cherenkov
counting, and compared with the amount.i.npat into the
reactions. Filter-retained RNA was extracted with phenol
and chloroform, antI isolated by ethanol precipitation in the
presence of 1’-2 pi; glycogen.
The istdalnil RNA was subsequently used as a tentptale
for avian myelothiasnamsi.s virus reverse lranscriptase (AMy-
tU’ Life Sciences) to obtain ct)NA. One hundred pmotes of
the 3 -printer (FiG. 1) wins added to the RNA and annealed
by healing for.1 minutes at 70" C., followed by chilling on
ice. "c5016 nnaction contained 5 U/SMV-ItT, 11.4 mM each
of dN1’Ps, 51) mM ’this-lit/I (pH 8.3), 60mM PInt/I, 8
Mg(OAc), arid 10 mM lYfI’, which was incubated for 45
minutes at 4$" (.’Ile cI)NAwas amplified by 1’Clt with the
S’- and the 3’-primers (110. 1), and the resulting 1)31/S
template, was transcribed to obtain RNA for the treat round
of SELLIX
To minimise selection of undesirable rritrtnceltamltxse-

binding seqireanctas, beginning its rotund thSreec we prc-rtoakrad
pools- with uitrorxllelosc IlIten before incubating with the
target protein-1hi, treatment worked welt to control back-
ground binding and helped ensure titirt each SELEX round

US 6,344,321 131

Lion of the components of this invention, tkc following acbirwid, In the present invention. (tar. SllLEX methodology
definittas are provided: is employed to obtain nucleic acid ligands to R(W and
. "ntictiiictcidjjs.anttn-naturaJfgc-cneL
occumn1, rerU s. acid having a,1’.rrabli. actitin on targo.- th The SELEX methodology is described in the SLILX Nnctmc&ithLcfoisrrTrrta.’ajgamj’. Jb18 Patent Applications.

rTfcad -inicith y--witlr-nuvieic--nckl "SELEX target" or "target" means any compound or tigarirl diruiigtiot this_appLic molecule of interest for which a Irgatid is desired. A target
at is sot lnmtrd to, binding of the target, cain- can be a proteia, peptide, carbohydrate, polysaccharide-,
(ytically changing the target, reading with the target in a glycoproteis, hormone, receptor, antigen, antibody, virus,
way which modificsfaticis (tar target or the functional activ- iii Substrate, tuelubolita, transition stare analog, colactor,
by of the target, covaluntly attaching In the target as in a inhibitor, drag, dye, nutrient, growth factor, etc. without
suicide iubibitur, facilitating the reaction between the target limitation. In this application, the SELfiX targets are HGF
and another motecithe In the preferred einbzxlitnenl, the and c-met. In particular, the SELEX targets in this applies-
action is specific binding affinity fiir a target molecule, such Lion include purified hOP and c-met, and fragments thentoL
target molecule being a three dimensional chemicalstructure is and short papikhes or expressed protein domains comprising
other than a polynucfr.otide that binds to the nucleic acid LIfW or c-met. Also includes as targets arc fusion proteins
ligartd through a mechanism which predoniinaittly depends comprising portions of HOP or c-met and other proteins.
on Watson/Crick base pawing or triple helix binding, As used herein, "solid support" is defined as any surface
wherein the nucleic acid ligand is not a nucleic acid having to which molecules may be attached thrciugli either covalent
the known physiological function of being bound by the us or ron-covalent bonds. This includes, but is not Limited to,
target molecule. In tire present invention, the targets are membranes, microliter plates, magnetic heads, charged
c-met and I-lOP or portions thereof. Nucleic acid ligiuid.c paper; nylon, tangrntiir-&tdgett films, functiotialized glans,
include nucleic acids that are identified from a candidate germanium, silicon. FifE, polystyrene, gallium arsenide,
mixture of nucleic acids, said nucleic acid tigaud being a gold, and silver. Any other material known in the act that is
ligand of a given target, by (lie method comprising: a) zs capable of having functional groups such as amino,
contacting the candidate mixture with the target, wherein cattxutyt, dm1 or hydroxyl incorporated on its surface, is
nucleic acids having an increased affinity to the target atsmscnntemplated. Thisincludessurfaces with any topology,
relative to the candidate mixture may be partitioned from the including, but not limited to,spherical surfaces and grooved
remainder of the candidate mixture; h) partitioning the surfaces.
increased affinity nvcleic acids from the remainder of the so As used hetein,’flGF" refersto tiepatocyle growth factt/
candidate mixture; and a) amplifying the increased affinity scatter factor. Tha includes purified hepatccyte growth
unclek acids to yield a Iigand.cnricbed mixture of nucleic faclorhcafler factor; fragments of hepalocyte growth factor/
acids, scatter factor, chemically synthesized fragments of hepato-
As used herein, "candidate mixture" is a rttirrtut’e of cyte growth factor/scatter factor, derivatives or mutated
nucleic acids of titTering sequence from which to select a 15 versions of hepatocyto growth factor/scatter factor, and
desired higaod. The source of a candidate mixture can be ftisiort proteins comprising Itepatricyte growth factor/scatter
from naturally-occurring nucleic acids or fragments thereof, factor and another protein. "HGF" as used herein also
chemically synthesized nucleic acids, enzymatically syntlirt. inelttdes hepalocyte growth factor/scatter factor isolated
sized nucleic acids or nucleic acids made by a combination from species other than humans.
of the foregoing lechniqtres, to a preferred embodiment, at As used herein "c-met" rc&m to thin receptor (tsr R(W
each nucleic acid has fiscal sequences surrounding a ran- This includes purilhtid receptor, fragments of receptor,
tiomized region to facilitate the amplification process, chemically synthesized fragments ofreceptor, derivatives or
A’ea.scd herein. "uce(carucrd"tnesnt etth r DNli,,I1NA, mutated versions ofreceptor and fusion proteinsrxirnpnsumg
inglustthtnrkmhlc-atrarekniu&anychendcaimrzti- the receptor anti another protein. "c-met" an usual herein also
hacattoheredfMotiultcatrdiis Ii9HisIit hut -ire not limited to utchudes the HOP receptor isolated from a specks other than
frt.those wtueti pr ve,ta-nther dhcrncal groups that mnrarr huniaris.
parate-addrrmonal eh,rr1,e polanitibtlrty hydrogen bonditig Note that throughout this application, various references
blcctrrrsiatir hiteractiar, to-fbc are cited. livery reference cited herein is specifically incor-
tigatidixm.cttr To the sackie aci&tigaisi ataa-whoic. Such poraleal in its entirety.

mull ffditialhiuietud biit i--nix tr eth to 2postttinct A. Preparing Nucleic Acid tgands to llG and c- aagai=arndalmsiiun,c=5-pt-i.ntanrr--pyrrmidtnc modilicatrorna, met gponiiars piJritre ruralticaltoimia. moiti1hcatinnsst-vsrrnvchtc-

ni5stiadtidb1tii4Titiiebsiuiiilii/ncif. In the preferred embodiment, the nucleic acid ligands of
rhromo:Joru:S.ind,s-araeif,-1aaekts’ria iscidif.catioiss, the present invention are derived Loon the SELEX method.
wetbyhntioessnus.ral-turarhb5,OlOgY. The SELEX process is described in U.S. patent inbinacs isocyltitine and iurgaaniilrne anal the hue. Sloditi- -----pplacalaots 8cr. No. 071536.428. entitled Systematic Hunk,-

is can atv Zion of Ligands by Lxponential Lnribrnent now abandoned, US. Pat. No. 5,475,09fl entitled Nucleic Acid
"SIiLIiX" methodology involves the combination of Ligands. att U.S. Pat. No. 5,270,163 (see also WI)
selection of nucleic acid ligands which interact with a target its 91/111813) entitled Methods for Identifying Nucleic Acid
in a desirable minuet, forexaniple hinting to a protein, with Ligand.s. These applications, each specifically incorporated
amplification rat thrice selected nucleic acids. Optional itera- herein by ruicretace, an, collectively called the SELEX
live cycling ofthe selactioniamplittcatiomisteps allowssetea- Patent Applications.
tierS of one err asniall number nt nucleic acids which interact The SLil.IIX process provides a class of products which
most strongly with the target from a port which contains a to are nucleic acid motuculmis, each having a unique sequence,
very large orinther of nucleic te.idc. Cycling ofthe sclectioo/ and each ofvttich has ttteproperty ofhitidingspecitieally to
amplification porcedrame iccavitioucal until a.rctederi goal is aderrinnal tarfirt cootptsuaad or molecule. Target molecules are

US 6,344,321 BI

preferably, proteins, but can also include antorag others
carbohydrates, peptidoglycaos and a variety of small mol-
ecules. SELEX methodology can also be used to target
biological structures, such as cell surfaces or viruses,
through specific interaction with a molecule that is an
integral part of that biological structure.
In its most basic form, the SELEX process may bedsalned
by the following stairs of steps:
1) A candidate mixture of nucleic acids of differing
.sequence is prepared. The candidate mixture generally
includes regions of fixed sequeuom (i.e., each of the mem-
bers ofthe candidate mixture containsthe same sequences in
the same location) and regions of randomized sequences.
The fiscal sequence regions aria chosen eillten a) itt assist in
the amplification ar,e1ms described below, (b) to mimic a
sequence known to hint to the target, or (c) to enhance the
concentration of a given structural arrangement of the
nucleic acids in the candidate mixture. The randomized
sequences can be totally randomized (in., the probability of
finding a base at any position being one in four) or only
partially randomized (e.g., the probability of finding a base
at any location can be selected at any level between (land
100 peseent).
2) The candidate mixture is contacted with the selected
target under conditions favorable for binding between the
target said members of the candidate mixture. Under these
circumstances, the interaction between the target and the
nucleic acids ofthe candidate mixture can be considered as

forming nucleic add-target pairs between The huger and
those nucleic acids having the strongest affinity for the
target.
3) The nucleic acids with the highest affinity for the target
are partitioned from those nucleic acids with teaser affinity
to the target. Because only an extremely small rtunrber of
sequences(and posslhly only one molecule of nucleic acid)
corresponding to the highest affinity nucleic acids exist in
the candidate mixture, it is generally desirable to sail the
partitioning criteria so that a significant amount of the
nucleic acids in the candidate mixture lapproximatnly
5-50%) are retained during partitioning.
4) Those nucleic acids selected during partitioning as
having the relatively higher affinity for the target are then
artaplilted to create anew candidate mixture that is enriched
its nucleic acids hiving a relatively higher affinity for the
target.
5) By repealing the partitioning and amplifying steps
above, the newly formed candidate mixture contains fewer
and fewer unique sequences, and tist average degree of
affinity of the nucleic acids to the target will generally
increase. Taken to its extreme, the StiUiX process will yield
a candidate mixture containing one or a small number of
unique nucleic acids representing those nucleic acids from
the original candidate mixture having the highest affinity, to
the target molecule.
The basic SELEX method has been modified It, achieve a
number of specific objectives. For example, U.S. patent
application Sea. No. 07/ttfitt,t193, tiled Oct. 14, 1932, now
abandoned, and U.S. Pat. No. 5,707,796 both entitled
"Method for Selecting Nucleic Acids on The Itasis of
Structure." describe the use of the SELEX process in con-
junction with gel eteatmphorrrais to select nucleic acid
molecules with specific structural characteristics, such as
bent DNA. U.S. patent application 8cr. No. (18/I 23,935. tiled
Sep. 17, 1993, entitled "Photcsehrictiuo of Nucleic Acid
I.igamtds,", now abandoned, U.S. Pat. No. 5,763,177 entitled
"Systematic Evolution or Ijgands by Exporsiatiat Enrich’

10

merit: Photaselectiota of Nucleic Add Uganda and Solution
SELEX" and U.S. patent application Sec. No. 09/fr93,293,
filer) inn. 8,1998, entitled "Systematic hivolutirinof Llgaaids
by Exponential Enrichment: Pttotoselectioa ofNucleic Acid
Ligatrds and Solution SIILEX" all describe aSELEX based
method forselecting nucleic acid ligands containing photo-
reactive groups capable of binding ant/or pholocrosslinking
to and/or ptiotoinaetivating a target molecule. US. Pal. No.
5,580.737 retiitieal "High-Affinity Nucleic Acid Ligands

That Discriminate lletwcen Thuophyllitte and Caffeine,- describes a method for identifying highly specific nucleic
acid ligatids able to discriminate between closely related
molecules, termed Cantriter-SIiL13X. U.S. fiat. No.5,567,588
entitled "Systematic Evolution of Ligatttls by Exponential
Enrichment: Solution SIiLEX," describes a SELEX-bascal
- inerliod which achieves highly efficient partitioning between
nhigouucleotidsa having high and tow affinity for a target
molarcute. U.S. Pat. No: 5,496,938 entitled "Nucleic Acid
Liganets to llIV4tl’ and lI1V-1 Rev," describes methods for
obtaining improved nucleic acid ligands after SELEX has
been performed. U.S.Pat. No. 5.705,337 entitled "System-
atic Evolution of Ltgatrds by Exponential liuriclnrnentr
Chemi-SIiLIIX," describes methods for covalently linking a
Ilgand to its target
25 The SELEX method encompasses the identification of
high-affinity nucleic acid ligands containing rrtodllled nucle
otides conferring improved characteristics on the Ilgand,
such as improved in vitro stability or improved delivery
characteristics. Examples of such modifications include
se chemical mhmons at the ribtise and/or phosphate and/or
base positions. SELEX identified nucleic acid ligands con-
taining modified micleotideit are described in U.S. Pat. No.
5,660,985 entitled "Nigh Affinity Nucleic Acid Uganda
Containing Modified Ntacleotides," that describes ndigo-
35 nucleotides containing nucleratiele derivatives chemically
modified at the 5-and 2’-positios of pyrimitlines. US. Pat.
No. 5,637,459, supra, describes highly specific nucleic acid
ligands containing one or more niacleotides modified with
P-amino (2-Nil3). 2’-ttuum (2’.P). and/or 2’-0--tnerhyt (2k-
40 tiMe). U.S. patent application Set. No. (W264,(Y29. filed
Jun. 2), 1994. entitled "Novel Method of Preparation of
Known and Novel 2’ Modillcd Nucleatsidesby lntramolectr-
tar Nucleophilic frisplacensetir," now abandoned, describes
uligonucleotides containing various 27-modhlied pynim-
tatmnes.

The SRL11X method encnnipasscs combining selected
oligonuckolides with other selected nligooucicotiakn and
rum-oligonu.cleotide functional units as described in U.S.
Pat. No. 5,637,459 entitled "Systematic Evolution of
ao Uganda by Ifirpririenlial linrichiment: Chimeric SLiLEX,"
and U.S. Pat. No. 5.683,867 entitled "Systematic Evolution
of Ligtcnda by Exponential liexuchuaent: Blended SELEX,"
respectively. These applications allow the combination of
the broad array of shapes and other properties, and the
efficient amplification and replication properties, of ohigo-
nucleotides with the desirable properties of other molecules.
In U.S. Pat. No. 5,496,938 methods are described for
obtaining improved nucleic acid ligands after the SEl.EX
process has been performed. This patent, entitled Nucleic
oat Acid Ligands In, L-tlV-ITT and 1EV-i Rev, is specifically
incorporated herein by reference.
One potential problem encountered in the diagnostic use
of nucleic acids is that oligonucteolides in their phospbodi-
ester form may be quickly, degraded in body fluids by
it intracellular anti cstracdllular enzymes such as endonu’
ceases and esasnucleases before the desired effect is mani-
fest. Crarlain chemical modifications of the nucleic acid

US 6,344,321 Bi
15

had a positive signulMoiw ratio. The progress of SELEX was monitored by nitrocellulose lilter-binding analysis of
the euricked pools (see below).
Truncate SELEX was performed by the hybridization
method described in US. patent application Set. No. 091275,
850, filed Mar. 24 1999, entitled "flaa Truncation SELEX
Method". incorporated herein by reference in its entirely.
Briefly, 2-F-RNApootswere body-labeled during transcrip-
tion and cleaved by RNusell using specific cleavage primers
to remove the lined sequences from the SIILEX pool (FIG.
2). This RNA was then bound to target protein HOF and
recovered following partitioning as in a conventional filter
SELEX experiment. The recovered RNA was then biotin-
lyated at its 3-prime uri and hybridized overnight under
appropriate conditions with single-atranded full-length
complementary strand DNA obtained from the starting
SELEX pool, front which the RNA had been mrsarscnlred.
The RNA!DNA complexes were then captured on
streptavidin-coated magnetic heads and extensively washed
to remove non-hybridized DNA. The bound DNA in the
captured RNA/DNA complexes was then eluted by heat
denaturation and amplified using conventional SELIIX PCR
primers. lb onnapleac the cycle, the resulting DNAwas then
used as a transcription template for generating RNA to be
cleaved by IlNasefi, and used in the next round of truncate
SELEX.

For plain SELu a polystyrene well was pre-blocked In
4110 ttl of blocking agent for (it) minutes at 37’ C. The
blocking agent wasremoved and the rintired amount ofRNA
in 100 p1 binding buffet was added and incubated for 60
minutes at 37’ C. White, polystyrene breakaway wells
(catalog 0950-2965) used for partitioning were from VWR
(Denver, Colo.). The blocking agents, f-block and
Supedstock, were purchased from Tropix (Bedford, Mass.)
and Pierce (Rockford, IlL). respectively. The prnadsorbtiurr
was done to remove any nucleic acids which might bind to
the well or the blocking agent. The random and totted one
libraries were not preadsorhed to plates to avoid loss of
unique sequences. C-met protein was diluted in LIBSMCK
(50 mM HF.PS, pH 7.4, 140 mM NaCl, 3 mM Edt mM
CaC1, 1 mM MgCl,), and was adsorbed to polystyrene
wells by incubating t(Epl of diluted protein per well for 60
minutes at 37’ C. The wells were each washed with three
400 1d aliquots of lilT buffer (HBSMCK, 0.1% I-block,
0.05% limerr 21)). and then blocked in 400 p1 of blocking
agent for 60 minutes at 37" C. SIil.liX was initiated by
incubating 100r1 of RNA in the protein-bound well for 611
minutes at 37’ C. The RNAwas removed and the wells were
washed with 400 p1 aliquots of HIT hulice Increasing
numbers of washes were used in later rounds. The wells
were than washed twice with 4(k1 11 water. RNA bound to
c-met was ctuted by adding 100 141 water and heating at 95’
C. ford minutes and then cooled on ice, followed by reverse
Iratiseription.
Nitrocellulose Filter-binding
In binding reactions, RNA concentrations were kept as
low as pouslblrbclween 1 and 20 pMto ensure equilib-
river in conditions of protein excess. Oligonucleotides ware
incubated for IS minutes a137’ C. with varying amounts of
the protein in 43 p1 ofthe binding buffer. Thirty-two micro-
liters of each binding mixture placed on pm -wet 0.45 pm
nitrocellulose filters under suction. Each well was immedi-
ately washed with (IS nit binding heifer. The amount of
radioactivity retained on the fitters was quantitated by imag-
ing. The radivativity that bound to filters in the absence of
protein was used Ibm background correction. The percentage
of input uligunueleotide retained on each litter spot was

14

plotted against the corresponding log protein concentration.
The nonlinear Ieaat square method was used to obtain the
dissociation constant (1c nrferctsm.Iellinek, Lynott et a].
1993, Proc. Nail. Acad. Si. USA. 90:11227-.31).
5 Competitor titration carves were generated essentially as
a standard binding curve, except that the protein and RNA
concentrations were kept constant, and the competitor con-
centration was varied. Curtrpelilozs were also added at a
fixed concentration in binding experiments to increase stain-
to gertey for purposes f cotnparisrg pool binding affinities In
these experiments, the competitor concentration was chosen
based on the results from the competitor titration curves.
Molecular Cloning and DNA Sequencing
To obtain individual sequences from the enriched pools,
to we cloned the PCR products from the final SELEX rounds
using one of two blunt-end cloning kits, Pcriitctly Blunt
(Novagen, Madison, Win), or PCR-Script (Stratagem. La
Jolla, Calif.). Clones were sequenced with the AR) Prism
Big Dye Terminator Cycle Sequencing kit (Perkin-Elmer
at Applied Binsyaterris, Fester City, Calif). Sequences were
obtained from an automated ABI sequencer, and text flies
were collated and analyzed by computer alignment and
inspection.
Boundary Determinations
is Five-prime and 3-prime boundaries of RNA aplaroers
were cleterrriintd by the method ofpartial alkaline hydrolysis
as described (Jetlirtek, Green or a]. 1994. Biochemistry.
33:10450-6).

Cell Assays so Standard cell culture procedures were employed in the course of performing in vitro experiments to test aptanmer-

nmndinted inhibition of HGF activity. For cell migration
assays, mounlayerta of A549 (lung carcinoma) cells were

grown on the top-sides of Matiigel.coated litter inserts s.c (Becton Dickinson, Franklin Lakes, NJ.) in 24-we1l plates.
The cells adhere to the upper surface of the tilLer, which is
placed in growth medium containing HGF. After two days,
the cells are physically removed from the top surface ofthe
filter. The filter is then removed from the insert and stained
an with crystal violet. Since all cells on the top ofthe filter are
gone, the only cells that remain are those that are have
migrated to the bottom ofthe filter. In the presence offlaP,
significantly more cells at,, found on the bottom ofthe filter
compared to controls without I1GL
4s Oligouucleotidc Synthesis and Modification
RNA was routinely synthesized by standard cyanoethyl
chemistry as modified (Green, Jeflinek itt at. 1995, Client
Blot 2:683-95). l\vo-prime-Euoao-pyrimidine phosphots.
ratitlite monomers were obtained from flit. Scientific (Sari
sq Luis Obispo, Calif.); 2-lIMe puritan. 2’-O}I patrine, haxyl
amine, and the aftr polystymee solid support were obtained
from Glen Research (Sterling. Vs.).
.-Fbr-additioa-oL4OIL-PEG,JLNA.oilgoreers.-wereayiithe
szrdwimnmino4irdarr-athpanition1ltiswa
as.srobsequeatlyreaeted with NtIS-ester 40K-PEG aranufae
’lured by Stanirwatcr Polymers, Inc. (t-luntSville. Als.), raa3
pstritiFhy-EIPfrCtitt a rrvcrsegbyg.prepare.zulz,wn..
2’-O-,jgimeSuintittliio6
Determination ofwhich 2-011-pndnca can he substituted
so by 2’-0-ntetltyl-pttrine was done as described (Green 1995,
Client Riot. 2:683-95). Briefly. Saul ofotigouncleotides was
synthesized with a mixture of 2 -0-methyl amidites and
2’-Olt amidites at defined purina positions. The set was
designed so that each oligonuctentide contains a subset of
as pamrially-x,ubsiiiuted ponnes, and the complete set encom-
passes all parities. Each aptanlec was 5’-end Libeled and
subjected to limited alkaline hydrolysis fotl,,wed by binding

US 6,344,321 BI

17 to HGF protein at two different concentrations, 50 and 100

pM. Following binding, protein-bound RNA was separated
by standard nitrettllelose filtration. Bound RNA was recov-
ered and analyzed by high-resolution gel electrophoresis
The fragmented alkaline-hydrolyzed aptatasets which were
not exposed to lIOF were run to establish the cleavage

patterns ofthe unselected aptamers. Hydrolysis occurs only at 2’.011-purines. It a given position requires 2’.014 for

optimal binding to UHF, it appears as a relatively darker
band compared to the unselected aptamer at that position.
ltesislts_t-tUtt
Five 1161’ SELEX experiments were done in total. The
first three were done by conventional filter SELEX, while
the latter two were dune by the hybridization truncate
SELEX method described in U.S. patent applicalionSer. No.
09/275,850, filed Mat. 24 1999, entitled "The Truncation
SELEX Method", incorporated herein by rtfemrtee in its
entirety. 1161’ SELEX 1 was done with 30N7 2’-F-RNA for
thirteen rounds of conventional filterbitadirst. HGF SELEX
2 was done with BINS 2’-r-RNA for thirteen rounds of
conventional lilter binding. HGF SELEX 3 was done with
30117 2i.FliNA for seven rounds by spot filter binding,
followed by eight rorinals of filter binding. IJGF SELEX 4
was done by hybridization filter SELEX for three rounds,
starting with Pool from IIGF SELEX 1. [101’ SEL.IIX S
was done by hybridization filter SELEX (or three errands,
starting with pan1 IL from UHF SELEX 3. HBSMC buffer
was used in conventional SELLiX reactions, and in spot filter
SELEX, blocking agents were added as described in Mate-
rials and Methods.

RNA Pool Binding With and Without Competitors Heparin.
and I11NA

To evaluate SELEX progress, binding curves with puri-
fied UHF protein were routinely done with evolved pools
during the course ofthese experiments. Representativebind-
lug curves are shown for 1511’ SELIIX experiments 1 and 2
(FIG. 3). Thesa data were used to ascertain when a S1IUIX
was complete in that (tardier progress was not likely to ocelrr
by performing additional murtals. HGF SELEX I readied its
maximal binding by round 8, with a binding affinity of
approximately 01 nM (PlC.. 3A earlier mends and round 9
were examined in other eapethasertla). UHF SELEX 2
reached its maximal binding tsy round 10, with a binding
affinity of approximately 0.1 nM (FIG. SB). IIQFSELEX 3
reached its maximal binding by round 11, afterseven rounds
of spot tiller pualitinning followed by four rounds of eon-
veartionsl litter SIIIJIX (see FIG. 411). ASELEIX experiment
which was deemed complete was characterized by cloning
and sequencing (Ste below).
UHF, like other proteins which have large clusters of
positively charged amino acids, exhibits a high degree of
non-specific binding to polynnitattic compounds. For
eannaple. random RNA pools hind to 1161’ with tow nano-
molar affinity, similar to the ittitre reported for 1161’ binding
to beparin, a potyauinnic sulfated polysaccharide known to
have an important biological role in HGF function
(Zioncheck. Richardson ct al. 1995,.1 Biol Chew.
270:168714). Competition binding to heparin as well as the
non-specific competitor [RNA was done to provide an
additional means of avaluasing SELItX progress. We did this

because the binding of random and evolved RNA pools to 1161’ occurs in a high-aflinity range which makes it difficult
to monitor progress. In other wards, random RNA hinds so
well to 116]’ that the affinity enhancement of the evolved
pools may not be adequately assessed in conventional birtal-
lug experimnurrt.s in the absence of competitor.
RNA pools from H111’ SIil.1ll< 3 were subjected to
competition with laeparin (FIG. 4A). This experiment dent-

otsstxates that random RNAis considerably more sensitive to
competition for binding to 1161’ than are the evolved pools.
new data are compared to these obtained from a binding
curve with the same three RNA pools (FIG. 411). In she
a absence of heparin competition, binding oftendons RNA to
1161’ is nearly as good as that ofthe evolved pools, whereas
the heparin competition reveals that the evolved pools are
significantly diifcrent in composition from random RNA, In
asktition, while rounds 8 and 11 are indistinguishable in
in conventional binding curves, round Ii exhibits improved
binding based ott increased resistance to Iteparlo coanpeti-
riota. These data cmsmaiijbttted to the eltoire of round Ii as tire
maximally binding pool from which we cloned and
sequenced.
is A similar, but more paunoutrectl, effect was observed with
LENA as the competitor (FIG. SA). These data indicate that
the round It punt from UHF SWIlL 3 ate at least four
orders of magnitude more resistant to competition for bind-
ing to 1161’ Than is random RN,’.. From theta curves, it was
251 determined that 815) oM tENA is the maximum concentra-
titan at which complete binding of evolved RNA peisisth.
Therefore, binding curves were dote at thisIRliAcemncen-
tration to compare the binding of different evolved pools
(FIG. SB). These curves were useful in determining that
25 farther SELEX rounds beyond round U did not Improve
binding.
T5’pieal data from a similar Set of binding competition
experiments done for latter murals of HGF SELEX I are
summarized in Table i.

to Cloning and Sequence Analysis of HG!’ SELEXes 1,2 and
3

Following determination of pool binding affinities for
1161’, we aulijected the optimal SET pools to cloning and
sequencing in order to isolate and characterize individual
us aptamem Data from 30117 1101’ SELEXes I and 3 are
summarized In Table 2, including binding affinitiesfor many
of the aptanrers. Asimilar data set was generated far 30118
1161’ SELEX 2 (Table 3). Sequences from HGF SELEX 1,
2 and 3 are designated 8-seq. number, 10-seq. number, and
an Il-seq. number, respectively, referring to the total number of
SELEX rounds each cloned pan1 was subjected to.
Sequences were analyzed and organized into groups with
significant homology. Motilk were analyzed and predicted
structures were drawn in order to analyze key features
45 responsible for binding to hOP.
Inhibition of LlGt’-mediated Stimulation of deli Prolifera-
tion

1101’, while not potent mitogcn, tinessrirntrlatc moderate
proliferation of many cell lines, which can be measured by
so incorporation at "H-thyine We assayed the inhibitory
activity of 1161’ aptaniers by measuring their effect on
pto1ifratioat of human umbilical vein.indotheliai cells
(111’VECS), or monkey bronchial epithelial (4MI3r-5) cells.
Based on the binding data and sequence family analysis,
55 fourteen nptamels were chosen for analysis in vitro because
they bind to 1161’ with high affinity and are representative of
different sequence families. The sequences are shown in
Table 4 aligned by a rough consensus which contains bases
in common to several families. All sequences are 3(1147
an except 10-2 which is IONS.
FIC,F stimulates proliferation of 1IUVECs by about Iwo-
to-three-fold (data not shown). The initial experiment indi-
cated that aptamers 8-17,8-102,8-1404,,1-122,8-126, 10-2
and 11-208 were effective inhibitors of ll(iU-induced
so lIIJVEC proliferation with E values in the low naoorrroLrtr
range (FlU. 6). Aptaretms 8-113 and 11-222 were less
effective and 8-151 exhibited Little or no concentration-

630

US 6,344,321 BI

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depetutenit ialtithsi. The latter,il’auvatnou is quosistcrit 850, Illeal Mar. 24, 19W. untitled "The Truncation SIILIIX
with the fact that aptautcr 8-151 does not hind HGF with Method", incorporated herein by reference in its entirety.
high affinity and actually hinds worse than the random pool. Binding of IlNasell cleaved pools was examined to dolor-
Several approaches were labia to reduce the length of mine which were the appropriate rounds to use to initiate
aplamens ’which retained signiticant inhibition of 1161: I) 5 truncate SLiIJ.iX (data not shown). None of the lNase1t-
bonndaay dcterniinaticcns lay biochemical separation of par- cleaved evolved pouts was clearly superior to another in
tiahly hydrolyzed aprananrs 2) sealuernec motif analysis and binding to IIGV, therefore, the pools which had been pro-
educated guessing; and 3) trrincete SWIX. vioushy cloned were chosen to use in Irutaccite SFLEX. The
Ilountlaries and Truncation encouraging result from this experiment was that after
Boundary determinations were dune for a sebun of an R2niararlt treatment, the evolved pools bound better to HGF
tqntaancrs that demonstrated is vitro inhibition of HGF than did random RNA, suggesting that even in Itte absence
activity- (ithrgastndard alkaline hyrtndysia procedure with ofthe fixed regions, sigaitkr.nat binding affinity wasretained.
5’-etsd-labelnrd KNA, we examined lIce 3’-boundaries; of This observation was salThrircntt evidence to suggest that
847, 8-102, 8-104, 8-126, tO-I, and 10-2. Additionally, truncate SELEX could enrich foe sequences which bound to
Y-eed4rdrelnd RNA was used for 5-1,oundary experiments is I!G} in the slaccuce of fitted regions.
with 8-17 and 8-102. These experiments were mostly Three rounds of hybridization truncate SELEX were, done
uninformative, probably because the high degree of tins- in parallel, using as starting pants tIGht Slit liX I round S
spectfic binding of RNA fragments, regardless of situ, and UGF SE! 3 round II. The truncate SLILEX rounds
absented the blinding oftruncated higlt-affinity aptarners to were done at equl-molar RNA and protein, starting at I eM
flGl Nina-specific binding of virtually all fragments gave an and decreasing to 0.5 and 0.1 nM. Signal-to-noise ratios
no boundary information, and reducing that protein concert- were very high during selection. Subsequent manipulations
 nation did not help. Instead, we tried to use pelyanionie were satisfactory even though the amount or recovered RNA
competitors (RNA and heparin to eliminate nonspecific was sub-picanniutar.
binding to reveal the actual brnandariea. The competitors To evaluate the progress ofthe SI1LEX, binding affinities
induced naon-spucilic binding, and 1KW was predominantly ta oftruncate round two and three were detcmtinedcttmpaavd
bound only by full-length aplamcxrs, revealing no boundary to those of the RNaseH-cleavedatrntllag pools (FIG. 9). For
information beyond the possibility that full-length aptanrers both SLl.trXes, the third round pacts bound with improved
are strongly ptefetred affinity for HG1 compararit with the earlier rounds.
The ante exception was açatatner 11-102 which had a tntcrestingly, The second rounds did not ktitSd llGF better
plausible 3’-boundary between two possible endpoints Sn than the staring material. The dissociation constants for the
which matte sense with respect to compurer.prediclad 5mm- third tinned truncate SELEXpciols ate 1-2 nM, representing
tunes IA). Itactud on that boundary data and asniclucal a 2-3 fold iartprovetncnt. While the magnitude of this
data, two truncates of8-102 were s atiresizeel and analyzed improvement not large, it is probably significant since
for binding ao 111W The sequence of(be full-length splinter 1KW as a target did nor easily yield affinity enrichment,
and the two truncates are shown, with fixed regimes under- 35 probably because of its intrinsically high affinity, for RNA,
lined: The two pools were cloned and sequenced, and binding
affinities were tktcrtnirced (Fable 5) The truncated niptamer

with the tsi.st binding TrSt, is among several sIns SSQ 11) N12 sequences which are niovcl that is, they were net found in
memetneangrgutreafomsgnaraqusro l,tuund Set] tO StOuLt at the attonesanrqiaenccd from the full-length Slit fiX pools.

emergence of novel sequences suggests that the truncate
ç zsrnenp saco tan NO.-14 SELIIX suet-tented in amplifying aptarners which write rela-
tively rare in tine full-leogLh.poobs. Aptamcr Tr5h appeared
In blotting it, l(GL thu 36xnerbuunnl almost as well as the mote frequently than any rathersequence,cons temat with the
fcafl-leiagth aptarusir, while the 28rner hottcrrl no better than as observation that it has better fnirtcliaag affinity any other
random 30147 (11G. 711), suggesting that the boundary data truncate. Other sequences which appeared multiple timers
were correct, also tend to be these with hintlhrtg affinities near or better
Truncation fly Sequence Structure Prediction than the pool K, of 1-2 nM
Several attempts were made to base truncatIon on motif
analysis and predicted smacratcas but those did not succeed an
in producing truncates which retained binding to 114W For The 36nrer derivative of aptamer 8-102 described above
example. alatamcn 8-17 folrktd into a reasonable predicted was tested for inhibition in vitro in a 4MIlr-5 cell pmtifuta_
structure which suggested two ealavhsas points oftruinealior, (iou assay (FIG. Ill). Although the 36mer retained high-
from its 3-prime temcninus, 38nrrntr or2itmer(tttG. 8A1 affinity binding to ItOh, it did not retain inhibitory activity
fluwever, binding analysis revealed that neither of ttacase st in vitro comparable to its patent aptarater 8-102 and ttptamer
truncates retained significant binding to flOP (G 88). 11-17 (PIG. 10).
These data suggest cither that the predicted structure is to carrier to itnprovn the activity of the Idmer, we loaned it
incorrect or that some ofthe 3-prienereghsin past base 38is In a fiwatrttirtioa with a YxfI’cap and 5’-40K PEG. The
critical for high-aflinicy binding of aptarnerS-17 tuIIGF. rnncntifrut aptianer, designated NX22354. was tested for
These two hypotheses are not mutually enrclnnctve to totiltastmoni t,f lit I mediated proliferation 4Mttr S cells
Nevertheless, we dad sat succarab no Obtaining a nieful (FIQ 1(A) 11cc data indicate that tine Shiner FEC aptansea
trwrn.-atc cr1817 by boundary and ’structural prediction- mblbats lt(rt and that it performs at least as well as the
Thincare SLI LX full-length apttraier’l 17 which had previously exhibited the
to ocdcr to generate arldctnc,nat tahort aptanteeni, we sash stnnuhest inhibition of all aptamers tested A. expected the
3uctcni advanced rccrandaofttte earL tSti[.EXustoaddrtional a stun PECvlated lttmerdtd rrtrt inhibit DOF unsgicstattg that
rounds of truncate SkI LX, trstnh the ircancarni a ’SELLX the Addition of PLC anchor the 1 -sap crantritaut.., to the
method destined in U.S.patent Application $ax Na (19/275 aptanter hni scttvnty The..epscrutseot was Also donu at

US 6,344.321 B

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krweraptasn,crnarrtcenri-.ntcrans,soppeartirag the proviocts result army of aptamcr-itnhihitors cylnakines and their recqaaurta
and showing wore clearly that 3nnor-PEG aptaancr is a and to be able to tailor contlainatircu trstatnlents for specific
helter inhibitor that the ti-Il fell-length aptainer (PIG. lilt). diseasat states.
Also tested by this assay was a nonbindiag apranner con- 2L0.trtethtytptirine Substitution of llGtcAptarnic, Nx22354
tainting a 3’-dT cap and 540K P11G. the VIi(iF apaamer s To improve the stability and pharnrcacokinctics of NX223
NXI83II,wlnielt had n.oebteca on lh(iFstimtalatiun (FI(; 12). 54, we determined which of the 17 2’-01 I put’inneca could be
In this same cxpenicttenr, a non-h’ltiiylated version of replaced. We did this by synthesizing, bar partially substi-
NX1838 arid the truncate SLiLEX aptamarTiSt were shown ruled 2-O.nncthyl.putine variants of the base sequence at
to have can inhihitcnryetfecr on lIt.lc(l’lli. 12) flnissut,geat.s NX22154 followed by analysis credracrihed in Materials and
’line that TrSI, similar to the Mulct haire uptamer oft’4X22354, as Methods. four partiatty-sncta’ctitntcet otigeaniucl.accklci
may require 5’-40E-t’IIG to tmthibtt 1161’ function. were Synthesized with a 1:1 ratio of 2’.0-methyl amidite:2’-

Oil areiclite (’Table 7). ’line data analysis nneastaccs the ratios lttlnlhitiuo of IIGE-rnediatecl Stimulation of Cell Migration ofthe sn.ulenansd to uasnctnictnd RNA at each substituted prarirtu HGF readily stimulates cell movement, Inemanint the name, position, based on qnsantitsniom of lacamida frocu tine gil. The scatter factor. We assayed the inhibitory of HOP data are summarized by position (PI(;. 15). At each position, açttatoers by measuring their effect ian A549 atehl migration IS the three unsulanlituted aptatenets Provide an important access a Matnigel coated membrane with 80 micron porusas comparison, which is cptessed as an average of the Iheate described in Materials and Methods (Table 6). ’lice ntnxsubstituttsd aptamens with standard deviation repncserstetl

112(22354 aptacnter fully inhibited HC.F-mediated migration by the error hats. Points that occur at ratios higher Than that at bath t and (1.2 pM ctnncerttratiorts, hut at 604 pM. (Inc of The nearby positions ate likely to requite 2 -Ott for
ellect was negligible. The monoclonal antibody control act binding.
(sample 3) was ntoderaw)y nilFective at the t pg/mI dose. The data strongly indicate that two positions, GS and P.25,
which is above it, publisited l!C, value taf{t.t-O.3pgfrnti for do not tolerate 2’-OMe substitution. two other positions, AS
inhibition of 4Mflr-5 cell proliferation, and (110, show a slight preference alcove the standard
Combined Inhibitory (lifect of hOP and VEnt Aglamners deviation of the utrseleirted RNA.
on HUVEC proliferation as The set ofOMe aptaoanrrs were also examined for binding
It was wpPnd that VEOF and HOP have an additive to itGF(data not shown). The taiuclittg data indicate that the
stimulatory effect on IICVIIC proliferation (Van Delta illlllt, OMel and OMe3 hind as well as the parent trrcsutnstitctred
Circulation. 97:3111-90), We observed this effect when Stimet, whereas 4L)Me2 and t)Mc4 bind less well. This
VEGF and tlGF were added, singly and in combination, to suggests that the substitutions in OMe2 and 0Me4 are less
LIIIVliCs, and we measured incorporation of tit-tltyntidine ri well tolerated with respect to 11611 binding in solution,
(FIG. 13). As expected, stimulation by HGF was relatively consistent with that fact that OMe2 and OMc4 are sulastituroci
weak compared with that of VEOF and together, the stintu- at P.25 and t15a respectively.
latory effect was greater than that elicited by VE(W alone. To confirm these rtasrtlt, two aptamntrs were synthesized
Based on these curves, we chose to add each cytokine at which are fully 2’-0-rrrcthyt substituted at that apparently
lit tag/mt for optimal stimulation in the aptanter inhibition as well-tolerated positions. That scqruette-es are shown below,
ecqxcrimaants. We then rested the effect of adding cane or laottt with tine 2’-0I1-pturinesslacrwni underlioed. MI other purnies
aplauters to the doubly-stimulated tells in the pre’snulacc of have 2’-01Ve and the pyritnidianat are 2’-llunjro solisrituted.

Inrath growth factors (FIG. 14A). We observed that each 4atsati 1 -OIL C.65etACGctcCiC6ACiVrK5UurtUt;

aptamcr partially inhibits the stimulation and that both Atur.snJcAmJcusuer: alto its 5Oct55 aptameas restalt in complete inhibition- Interestingly, the aas,.t, a..oa oosng,Mar,crarccr,Acatcracs"tcc.t.trr magnitude of the iatltibutoey caffatet of’tacit aptauiacraannghly AV-fjUCAVCXCC_-C 5110 tat 5Oct57 corresponds with the magnitude ofthe stiratnlatinneocafernst!

by each cytcakitlat. This observation sttgg.ncsts that tine snionta- Soiluence 4xScrb 2-0I1 contains all four of the 2’-Olt-
lacoryetfectdfcaclniyuokine can be inhibited independently. pnnnnos inquestion. while 2icStah, 2 -Oil has only the two
and that the two cyaoldnes stimulate HUVIICa iudepen- 2’-DIl-ptarittncs most likely to be required.
dently. Binding of these oligocrrers to 114W was esanuirned atom-
’lice remaining, two panelsof 140. 14 (HG. 1411 and I’Ui. pated to the uusubstittsted parent and that bully 2’.0-nactlayl
14() are controls in which cache ntaakine being adrninisleced sctbstittciecl RNA (FIG. 16). Based on these binding curves.
separately, demonstrating that the lIOF and VEGI- aptanners NX22354 totara 2’-()Mc substitution te all ptirincs except
do not cross-maci. that is, each aptrrmnaer affects onlythe
-

65 and P.25 (aplanner 2xSub 2’-OH) with minimal less of

binding cyaoldnc Against which it was selected. For the fiCnl5uatimu- affinity. The other two positions in question appar-
hated cells, we observed inhibition by the IIUF aptanner ently are not tcatnsirerl to be 2’-01l sirton atnanter 4xritsts
NX22354, hint not by alts VtiGl- aptamer NXt8Sht 119G. 2’-Ot-1 binds no better than aptatnnee 2cmSttla 2’-Ctl 1.
148). Conversely, stimulation, by VI1OP was inhibited by lteo a3aectmets have been synthesired With 5’-lttK-PEG
the VIIGF aptzrnaerNXl&38, bat-was unalfecterIby the HOP ss and a 3’.dTcap: one is (ally 24)-me1byl stnbstittshcad and the
aplamer NX22354 (FIG. 14C). other contains T-011 at positiocac 65 and A25. One ofthose
These data, along with the fact that HOP, like VIIGF, is an will preaumb1y supplant 11X22354 as the lead tttP
angiogenesis factor intake it intriguing to consider dual aptanner for further testing in vitro and in vise,.
administration of VIGP and 114W aptamets to (teat tttnsona. Resultse-n’.et
lturilserntore.the availability ofaptattaanswhich inhibit other en c-Met SElt.EX
growth factors suggests further crrcnbittatisarcs of the VtKil In the c-Met plate SEA EX experiments, the concentration
or the hOP aptarner in ccurataioitiou with other aptanters, liar of nucleic acir!swa.stowered initially, butt then raised in later
example, aptamers than inhibit 1cPGF, platelet-derived rounds so that the ratio of tine,cnarlelc acid to prdt.cisa would
growth factor (tSXitt), transforming growth factor beta he very high. Thin was done in order tct:create conditions of
(TOF), kcratinncyte growth factor (flOP), anal/or their is high stringency which may select for hirijanr affinity aptam
receptors allowing for the pecisihility that any ccnitaiicaction errs. Striccg.anscy was also applied by increasing the number of
o(these izhibitonsmavbeielevastThegotnlis it, hcaveacn washes,

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23
S1ILKX (’oci Binding
Binding of SELEX pools to

c-met was asstssed through
round

7 (fIG. 17). The butdingdata indicate that the SELEX

resulted in aboin a20 fold improvement in K4 from 20 UM to I nM for both ’a" (4(N 7) and "b" (30748) pools. S itcc the cmel protein used in SELEX is an tgG tbsio,t

protein, we tested random 40747 and round 7c RNA pools Cot
binding to human IgO, and
c-met. The binding dissociation constants obtained are as followst

TABLE

8

biedi.g,mi te,o,iatioe esu0ct,

SELItX ’anal Preci., it,,

nod,,, 1O, -1 pM
S, t5ct, 23 am
. edomc-meat lttr aM
7.c-set

2

24 not shown). Since Ig(l, aptatners have nut been isolated
fSom an Nit type library, this analysis was not done for the
30N8 SEtLEX. PCR of random and

c-met SELEX tound Is

pools yielded no signal after 20 cycles. However, rounds 2a,
3a, and 4a had steadily increasing signals that were easily
detectable after 10 PCR cycles. Thus tgG aptamncrs
appeared relatively early in the 40747 SELEX experiment
For

a negative control, PCR was done with

a nucleic add
lo pool from

a SELEX known to lack tgG, aptamers. For
positive controls, PCE was dune with pools from either an
Ni-based lgG,, or CTIA4-lgG, SELEX. ZgQ, aptamers
were first isolated from both of these SELEXes. The nega-
tive control had no detectable ISO, aptamers aftcr 20 PCR
’- cycles. The positive controls bad ticteclabie signals after 10

PCR cycles. C-nit Aptf1rers
The sequences of 19 clones from round 7c-40747 fail into
The affinity of round in RNA for both IgO, and

c-met 2a The

families with two sequences each,

a group with thee
proteins improved about 50-6olcl. There ate several interpme- unrelated members, and six scquctlmms closely related to
tations to this tenth. Aptameis may have been selected known lgO aptamersequences(Table 9). Thus, at least

6 of

which bind with better affinity to both proteins. This the 19 clones (32%) arc human IgO, aptamers. This mm

- assumes that the difference in binding between lgG and dents the results of previous analysis that indicated line c-met is due to C-met specific aptanmers. However,

the

IWO presence of were made in different cell Lines which may have igG aplanrers in this SELEX experiment.proteins
different glycosytation patterns which could influence bind- Ofthe 13 clones sequenced from round 7h-30N8, six are
lag. Thus, ifthe differences in affinity are due to differences almost identical, another five are closely related, and two arc
between the free 1g0r protein and the lgG,, domain in

c-met, 3t, distinct (’fable 10)
then there might he few if any

c-metspecific aptamersin the
round

7 pool. Nine clones were tested for binding to

c-met nrlWl&, six

In order to address (bean issues further, random and round from the 40N7 series and three from the 30N8 series. These
S RNA pools from both libraries were examined for binding clows were chosen for the following reasons. Clone 7b4 is
to the

c-met and 1(011 proteins (FIG. 1$). Roth of these the most frequent clone in family

1 and is representative of
proteins were oracle in the same cell litre and contain the almost all of the sequences isolated from the 7b-30N8
same lgG,-His,,sequence. Random IlNAfrom both libraries library. Cones 7b-10 and 7b-12 are the two clones from the

binds about the same to each protein (K,,s.-.59 riM). Round 71,,30y45 library that bad different sequences. From the 5 front the both libraries of c-met SELEX binds better ’° 7c-40N7 pool, the chosen representatives were: family 1 c-met than to 1(0K (-100-fold better for the 30N8 pool and an (clone 7c-I), family 2 (clone 7c4), family 3 (clone 7c-23), 3-fold better for the ’toNi pool). However, round S RNA family 4 (clone 7c-2.6), family 5 (clone 7c-25), and the pools do bind better than random RNA to XDR. These

results imply that, while there are probably aptamers which presumed tgGl family (clone 7c-3).
bindto human IgGor(1ilS), tag in the round

S pools, there Results are shown for ory two clones, including 7e-1
may also be

c-met açrtamrm 41 which wasthe only one r.bsekvcd to bind to

c-met better than

Detection of lgG Aplanrers By PCR 1(0K (FIG. 19A). Clone 701, which appeared twice in time
Another approach for determining if tgG1 aplamers are 401’47 series, may exhibit biphasic binding behavior with

a present in the SELIIX pools was to subject them to PCR. high affinity binding K,, of -50 pM and a lower affinity Predominant IgO, aptamers have been isolated from N7

type libraries which have

a Itatuwu sequence (iiitos l’agratis so hirtaliuti K,, of -5 aM. All eight other clones bound to 1(0K
and CImint, l)ang personal communication). For the PCR,

a as well as to

c-row, including 7c-3, which is shownhere as
DNA uligonucltotide: meptesmifltauve example (FIG. 1911). Clone 7c-3 and all
ML124; S-ArviAtrITtiniAAsAMaAAccotrUcar- others besides 7c-1 are presumed to be IgG, aptamers.
SIFQ to NWAR to summary, IWO clones (identical to ic-i) out of 32

was used that is complementary to time most prevalent s5 appaictrtly bind

c-met specifically and with high affinity. liar

747-series human Iget, aptantersequence, and differs by mily remaining clones appear to be lgG1 aptarnels. a few bases from most other igO, aptarnets. This Wit

primer is the same length as the selected sequence of the TABLE

I

major lgG so that it can tolerate mismatches and hybridize Biedi5 rttteits,

. (-I,-,FSmitESr

1 pod.

to similar sequences. fia ,,jth ’a,] stUart compatil,, ISMA.
The MI.-124

3 -primer. RNA past it,, (aM) it,, (SM)/IRNA ML-34i 5t5iCAO05CtArACUACçCA.

rEars-,’ stro it) t’tO tas ,as!oa, atts’i 1.5 55.1

ttot Satiot IttrUt 007 sic was used with a 5’-prim.mr containing the 17-promoter cs tar,,’ smtr.rx i tory ore sat sequence present in alt cloned aptamers to amplify 411747

series nucleic antis pools; random, Ia. 2a, 3a and 4a (data

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-continued

. 211. EaSGTfla 61

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(80) Priority Datac ARIPO patent (SE. LB. MW. SD, BK, UG), Eurasian patent
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MetlunIa for Identifying and prepating ho-afftn1 nucleic acid uganda to cytebinea and hat uganda obtelned thereby me dlaclontd.

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acidJigands-to okhnzahe method utilized herein for idenufying such nucleic acid ligands in called SELEX, an acronym for Systematic Evolution ofLigandsby Exponential enrichment This invention specifically includes methods for the identification ofhigh affinity nucleic acid ligands ofthe following cytokineer WN-gamma, lL-4 IL-H), INFer,

10 hetdiselosed-arA-ligds-tollN-ganmaI-IL-4-lL4OfandTNFu--Also disclosed RiO DNA ligands toP.ANTES. pecffi pie provided of

tpyxeoligonueleolides ofthe present invention are useful as
pharmaceuticals or diagnostic agents.
15
BACKGROUND OFTMINVEZT1ON

Cytoidnes are a diverse group ofsmall proteinsthat mediate cell
sigxralinglcommuthcation. They exert their biological fimctions through Upeciflo receptors expressed on the surface oftarget cells.
20 Cytokines can
uumunefhematopoietms, mterferons,tumor nccrostslactor(ThW)-retatethnoleculea-and
the sidkines.Rapresentalive immunedhematopoictins include eiythropoietin(EPO), granulocyte/macrophage colony-stimulating factor (GM-CSF). granulocyte colony-stimulating factor (O-CSF), leukemia inhibition factor (LW), oncostatin-M (OSM),
25 cilary neurotrophic factor (CNTF), growth hormone (OH). pzolaclin(PRL), interleukin (IL)-2, IL-3, IL-4, IL-S, IL IL-7, it-9 IL-lu, and JL-l2 itepresentalive ixiterferons
UN) include lFNec, IFN,and WN-gmnma. Representative TEF family members
include TNF a, interferon MN) p.e(CD40-L), CD27-L, CD30-L, and nerve growth factor (NGF). Representative chemokinesinclude platelet factor(PF)4, platelet basic
30 protein (PB?), grout, MK), ENA-78, macrophage inflammatory protein (MIP)!er, MIP1 p, monocyte chemoatiractant protein (MCP)-1, 1-309, HC14, Cl9, Regulated on Activation. Normal 1-cell Expressed, and Secreted (RANTES), and)L4

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isfundamental to the success or failure ofthe immune response.

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IPN-gamma plays

a majorrole in promoting inflammatory responses both directly,
and indirectly through its ability to enhance NP-

a production. During an inflammatory
response, cells leave the circulation and migrate to the point ofinfection. During this process they must first bind to and then extravasate through vascular endothelium. Both WN-gamma and NP-a can promote the expression ofoverlapping sets of cell adhesion molecules ([CAM-1, E-setecthu, and others) that play an important role in this process (Pober at al., 1986; Thornhill at a.., 1991). In fact, experiments have shown that these two cytokines exhibit synergistic effects in up-regulating cell adhesion molecules in vivo (Munro etal., 1989). One can envision microbial infections in which the microorganism is already widespread as the time the immune response develops or in which the response does not quickly rid the host ofthe infectious agent This results in continued

I cell

activation inducing both local inThmmation and tissue damage with ensuing loss of normal function. Indeed, when the infectious agent is oflittle intrinsic pathogenicity, the disease induced by the infection may largely reflect the consequences ofsuch

a response.

Excessive production ofWN-gamma may play

a rote in autoimmime disorders

review see Paul and Seder, 1994 and Steinman, 1993). The mechanism for this may
involve excessive tissue damage due to aberrant or enhanced expression of class

I and

class 11 MRC molecules or the role ofan excessive THI cellular response.

A role for

20 IFN-gamma and the tissue-damaging effects ofimmune responses mediated by Tilt-like cells has been suggested in autoimmune disorders such as rheumatoid arthritis(Feldmann, 1989), juvenile diabetes(Rapoport etal., 1993), myastheniaginvis (Ga at al., 1995),
severe inflammatory bowel disease (Krthn 1993), and multiple sclerosis (Traugott, 1988).
25
1L4

lutedeulun4IL4is aienhlyptoptccytokmneflrstrdentifiedin 1982 as
aD cellgmwth factor MCGD TgmqKd etal,J982) ititbat me was
identified asan igol enhancing factor (Isaka

a at al., 1982). Because ofthe effect IL4
30 has on

B cells, was first Called BCOF-1, latertanned BSF-1 (B-cell stimulatory
factor’l), and in 1986 it was given the name IL-4. The cDNAa for murine IL-4 (Noma at

W096/40717 PCT/US91V09537 6
al., 1986; Lee at al., 1986) and human IL4 (Yokota ci al., 1986) have been cloned,
sequenced, and characterized.
rL-4 can be regarded sadie prototypic member of

a family-ofimnunwie
(for

a review see

5 1991). This family consists ofIL-4, IL-5, IL-3, and gxanulocyte-macrophage
colony-stimulating factor(GM-CSF). The properties shared by these proteins leadsto this grouping and include, i) the linkge ofthe genes for the members ofthe family (van Leenwen etal., 1989), ii) the action ofeach member ofthe family as

a hemnatopoietjc

growth factor in addition to any effects it may exert on lymphoid cells, iii) the receptors
10 for these proteins are all members ofthe hematopoietin family ofreceptors(Bazan,
1990a), and iv) coexpression ofthese factors by

a subpopulatioa of cloned CD4

T cells

(the so-called Tic cells) (Moamsan el al, 1989) and by mast cells(Plant etal., 1989). The remarkable pleiotropic effects ofIL-4 are mediated through binding to cell surface receptors (LL-4R). The marine IL-4R (Mosely et al., 1989; Harada at al., 1990),
15 and the human IL-4R (ldzerda at al., 1990; (lalind at al., 1990) have been cloned,
sequenced, and characterized. IL-411 are present on

a variety ofhemaropoieiic (Park at al.,
1987) and nonhenmtopoietic cells (Lowenthal at at., 1988). On both human and murine resting

I and
B cells, IL4Rare present In low numbers (<400) and are regulated by
cylokines and otherfactors. The receptor binds IL-4 with high atflnity(Kd

= 10,10 hI).

20 Now that most ofthe receptors for immmmoregulatory and heniatopoietic cytokines have
been cloned, it is apparent that themajority ofthese receptors fall into

a large family. This

bematopoictic cytohine receptorsuperfamily includes receptors for IL-4, IL-2

(

b and
g

chains), IL-?, IL-9, and IL-13 which modulate the lymphoid system; and receptors for erythropoietin, granutocyte-colony stimulating factor(G-CSF), GM-CSF, IL-3, and IL-5
25 which modulate the hemopoletic system. The superfamily also includesreceptors for
factors believed to normally function outside the immune and hemÆtopoieticsystems, including receptors growth hormone (Gil), prolactin, leukemia inhibitory factor (LW). IL-fl, EL-1 1. and ciliary neuroimphic factor (CNF) (for

a review see Kishimoto
1994).
30
A general first step in the signaling processes ofimmune and henmatopoietic
cytokines maybe ligand-induced dimeriration ofreceptor components whose cytoplasmic

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regions interact to initiate a downstream signaling cascade. The IL-4 receptor has a long
putative intracellular domain (553 amino acids in mouse, 569 in human) with no known
consensussequences for kinase activity crier nudeotide-binding regions. The
biochemical nature ofsignalsinduced by the binding ofIL-4 to its receptor have not been
5 elucidated. It does appearthat the cytosolic domain ofthe receptor is essential for its
signaling function (Mcselyetal, 1989). Ligand induced dimerizasion ofthe IL-4 receptor appearsto be a critical firststep in LL4 mediated signal transduction.
isnsflmphytand
diftcrendation &am(Rabin et Was-first-
10

toigE3munmoglebulirrsCoaanØtaL, 1986; Lebmait and Coffinan, 1988, Del Preteel
al., 1988). The effect ofIL-4 on the to viva regulation ofIgE has been clearly
demonsuatrsL Neutralization ofIL-4 by treatment with a monoclonal anti-IL-4 antibody
15 (Finkelman at al, 1986) or amanoclonal antibody to the IL.4 receptor (Finkelman a! al.,
1990) will block the I8E response. A recombinant soluble IL-4 receptor bas been shown
to inhibit 1gB production by up to 85% In vivo (Sato et al., 1993). IL-4 deficient mice produced by gene-targeting in marina embryonic stem cells have normal B and cell development, but serum levels ofIgGI and IgE are strongly reduced (Kuhn et al., 1991).
20 IL-4 augmented 1gB production leads to an asopic state (allergy/asthma) (Finkelman et at,
1989;Katonaetal, 1991).
The LL-4 mediated up-regulation ofIgGI may play a role in the inflammation
cascade. IgGi has recently been shown to form immune complexes which bind to the cellular receptors for the Pc domain ofimmunoglobulins (Fda) leading to an
25 inflammatory response (Sylvestre and Ravetch, 1994. Ravetch, 1994). IL-4 transgenic
mice have been produced that bypesexprcss 1L-4 (Tepper etal., 1990). These mice have elevated levels ofserum lgCIl and 1gB and they develop allergic inflammatory disease.
These findings demonstrate the critical role IL-4 plays in the humoral immune response. Another major physiologic role for IL-4 is as a lymphocyte growth factor (Hu-Li
3D etal., 1987; Spits etal., 1987). 1L4 enhances the proliferation ofprecursors of c)totoxic
T cells(CTLs) and their differentiation into active CD8 CTLs (Widmer and Gtabstem.

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1987; Irene, 1988). IL-4 appears to augment the IL-2 driven induction of
lynqihokine-activated killer(LAK) cells (Higuchi etaL, 1989), which have been shown to lyse a variety oftumor cell targets without major bistocompatibility complex WC) restriction. The role played by JL-4 at this crucial nexus ofI cell effector function is
S fundamental to the success orfailure ofthe immune response.
IL-4 has been shown to affect noulymphoid bematopoietic cells in a variety of
ways. IL-4 has been shown to modulate monocyteimopbge grwth(Mcmnaes and Rennick, 1988; Jansen etat, 1989) while enhancing their differentiation and cytotoxic activity for certain tumor cells(Cra’wford,etal., 1987; Ta Velde etal., 1988). IL.sl also
10 has activity as a stimulant ofmast cell growth (Mosniamm 1986; Brown etal., 1987),
and increases production and recruitment ofcosinophils(Tepper etaL, 1989).

vneyofcellsrfaccmolecuIouiiryth-thverseimpJtea1rons4or, disease. Specifically, IL-4 can interact with tumor necrosisfactor (ThF) to selectively
15 enhance vascular cell adhesionmolecu1e-1jV
onflam(th4992a1
vrrth TNF or IFN-gamma has been shown to increase both MHC mtigen.and tumoraasocatedantrgenssmononavanetyofneoplasticeells-(Iloonctalj991)
As detailed above, IgGI immune complexes bind to the cellular receptors forthe
20 Pc domain ofimmunoglobidins (FcRs) leading to an inflammatory response. Inhibition of
IL-4 and the subsequent reduction in IL-4 mediated IgGl expression may prove
efficacious against immune complex inflammatory disease states. Indeed, inhibitory
uganda to JL-4 might also prevent the LL-4 mediated overexpression ofVCAM-1, thus attenuating the ability oil cells to extravasate at sites ofinflammation.
25 Inhibition ofIL-4 activity with a monoclonal antibody, a recombinant soluble IL-4 receptor, or gene knock-out, results in a reduction ofserum 1gB levels. An inhibitory oligonucleotide ligand to IL4 could be clinically effective against allergy and allergic asthma

A recent report has described a disorder in bone homeostasis in transgenic mice
30 that inappropriately express IL4 wider the direction ofthe lymphocyte-specific proximal
promoter for the Ick gene (Lewis etal., 1993), Bone disease in these mice resulted from

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markedly decreased bone formation by osieoblasts, features identical to those found in human osteoporosis. Inhibiting this LL-4 mediated reduction in osteoblast activity may prove beneficial against osteoporosis.
Graft-versus-host disease (GVHD) is a major complication ofhuman tissue
5 transplantation. GVHD does not exist as a single clinical manifestation but can involve
immunologic abnormalities ranging from immunodeficiency to systemic autoiminuniiies (Ferrara at 1991). These systemic autoimmimities include clinical and serological manifestations ofhuman systemic lupus eiythematosus (SLE). Several marine models of SLE have been developed (Gleichmann et al., 1982; van Rappard-van Der Veen at al.,
10 19821 and the induction ofsystemic GVHD in mice has been described (Via el al., 1988).
Two recent studies have shown in vivo efficacy ofa mouse monoclonal antibody to 1L4 in preventing GVHD and SLE in these murme model systems (Umland at ci., 1992;
Ushlyama ate!, 1995). These observations suggest that an inhibitor ofhuman IL-4 may
be effective in treatment ofchronic systemic autmmmuniiies such as SLE and GVHD.
15 A variety ofmicrobicidal infections are characterized by depressed cellular but enhanced Immoral immune responses, which suggests a Tw type ofresponse to infection.
This Tphenotype is characterized by T cell secretion ofIL-4, as detailed earlier. IL-4 blocksthe microbicidat activity ofIFN-gamma activated macrophages in fighting Letvhmania major infection (Liew etal., 1989; Leal eta!, 1993). Inhibition ofIL-4
20 would enhance theT. effector arm ofthe immune response enhancing cellular immunity
and leading to the resolution ofinfection. Neutralization of1L4 in vivo allows mice otherwise susceptible to Letslnnanw major infection to fight offthe parasite and clearthe infection (1{einzel at aL, 1989). Several informative studies have looked at the TNIIT,, phenotypic distinction in infected mice, and suggest aT,,, dominated response being most
25 effective in fighting microbial infection (for a review, see Slier and Coffiuian, 1992).

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IL-b
IL-10 is a W_ okincTroduced-b
cytokines not Th2
ai199lfln addition-to the effeetoILCD4cdlls1wthThLphenoipciL1abniflhjhjt
S 1CDST cellswi1hThl-1ikeILlThinirAfnit,,,,, Activation. It can-suppressff

macrophagodeaetivatorzo&aneffectiveanti-inflamatoygent-inaddi1L4o prevents the WN-g -induced-synthesis-of nWw-o)iid to
10 ntmcellu ’Ill at al., 1992).
Both human andmouse (kIL-lO and raiL-lU, respectively) have been cloned and
expressed (Moore etal., 1990; Vieira etaL, 1991). The two cDNAs exhibit high degree of nucleotide sequence homology (>80%) throughout and encode very similar open reading flumes (73% amino acid homology). Both proteins are expressed as noncovalent
IS homodimers that are and labile (Moore etal., 1993). Whether monomers are equally
bioaciive is not clear yet Based on the primary structure IL-10 has been categorized into
the four a-helix bundle family ofcytokines (Sbanafeltet aL,1991). Possibly due to high degree ofsequence homology and similarstructure hIL-lO has been shown to be active on mouse cells (Moore etal., 1993) but not vice versa. hIL-lo is an 18 kDa polypeptide with
20 no detectable carbohydrate; however, in mIL-10 there is one N-linked glycosylation. The
recombinant hiL-10 has been expressed in CR0 cells, COS7 cells, mouse myelonta cells,
the baculovirus expression system and E. ccli. The rIL-lO expressed in these systems
have indistinguishable biological behavior (Moore etaL,l993).
Parasitic infection often leadsto polarized immune response ofeither Thl or Th2
25 type which can mediate protection orsusceptibility. The outcome ofa parasitic infection
depends on the nature ofthe parasite and the host. The best understood example is Leithmamamajor infection in mice. L major is protozoan parasite that establishes an intracellular infection in macrophages, where it is mainly localized in phagolysosomes. Activated macrophages can efficiently destroy the intracellular parasite and thus parasitic
30 protection is achieved by macrophage activation. Nonactivated macrophages do not kill
these organisms. As expected, activation ofmacrophages upon IFN-gamma treatment

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12

enhanced the protection, whereas JL-4 and U,10 blocked the increased microbicida! IL-12 can also induce the development ofthe Thi subset By using Lysteria
activity induced by IFN-gamma(Liew of aL, 1989). In most inbred strains (example, an intracellular gram-positive bactesiuni, infection in antibody I cell
C57/BL6) cutaneous infection ofL major often leadsto localized infection with receptortrausgenic mice as a it has been shown that IL-10 can block the production
spontaneous healing and confersresistance to reinfection. However, in BALB/c mice, L of1L-12 fromniacrophages (Hsieh et aL1993). Thus an IL-i0-antagonist will tip the
5 major infection induces noz,rotective immune response by producing IL-4. The antibody 5 Thl/Th2 population predominantly to TM type environment by 1, preventing the
response mediated by IL-4 is ineffective and leads to death (Howard of at., 1980). In inhibition ofthe production ofThi cytokines 2 by allowing the production ofa cytokine
healing strains a strong TM response has been noticed with high level ofIFN- g, whereas that induces ofThI subset
in susceptible BALB/c mice a nonproductive Th2 response with significant levels ofIL-4 With experimental evidence in hand it has been proposed that the resistance and/or
was found (Heinzei etal., 1991). Furtherit was shown that a single injection of progression to AIDS is dependent on a Th11Th2 stage ofan individual (Clerici Shearer,
10 monoclonal anti-IM-garnma antibody can convert a resistance into a susceptible mouse 10 1993). This hypothesis is based on the findingsthat progression to AIDS is characterized
(Belosevic of aL, 1989). As expected, the treatment ofBALB/c mice with anii-IL-4 by loss ofIL-2 and IFN-gamma production (loss ofTh1 response) with increase in LL.4
antibody led to the development ofThi response and healing (Slier Coffman, 1992). and 11-40 (acquired Th2 response). Many (H1V-exposed individuals)
Thus, depending on the nature ofthe pathogen, changing the Immune response to a T cell generate a strong TM-type response. It isimportant to note that afterscroconversion both
subset with a protective phenotype can lead to therapeutic intervention ofthe disease state. IL-4 and IL-lB levels go up at the expense ofIL-4 and IFN-gamma. However, in
Understanding the regulation between the Thi and Th2 phenotype mediated by cytokines 15 full-blown AIDS patients, TM response seems to be mediated by high levels ofIL-JO but
will help in desig ingcytokine-antagonistintherapeutics. The production ofiL-ID is not with IL-4, the level ofwhich goes down to normal in these individuals. Mi anti-IL-Id
strongly increased in mice infected with various pathogens such as Leishmania major, reagent may serve as a potential therapeutic in shifting the TM response toThi in AIDS
Schistosoina mansoni, llypanosoma errol and Mycabacteriwn Leprae (Sher et at, 1992; patientsto offer protection.
Saigatne et al., 1991, HeinzeleraL, 1991).
20 When designing immune therapy to facilitate mounting the right arm ofdefense 20 TNFu
mechanism toward pathogens, it isimportant to maintain a balance between the two arms TNFs is an extiacellutar cytokine and a central mediator ofthe immune and
also. Th2-type responses may be important in controlling the tissue damage mediated by inflammatory response (Sculler of at, 1989; Vassalli, 1992). It is a homo-irimer (Smith of
Thl cells during the response to an intracellular infectious agent Keeping some Thi cells ci., 1987, Eck eral., 1988), and has a subunit size of 17 kD. It circulates at concentrations
functioning in a predominantly Th2 environment can help abrogate damaging effects of ofless than Spg/rnl in healthy individuals(Dinarello etal., 1993) and it can go as high as
25 ThI by secreting EL-10 and IL-4. One extreme ofthe spectrum ofTh11Th2 isreflected in 25 lOOt) pg/mI in patients with sepsis syndrome (Casey et al., 1993). The human INFO is
transgenic mice lacking the IL-JO gene (Kuhn et al., 1993). The IL-Id deficient mouse is nonglycosylaled, whereas in some other species (notably the mouse) glycosylation occurs
normal with respect to its development ofT and S cell subsets. However these mice on a single Winked site in the mature protein, but the sugar moiety is not essential for
develop chronic enterocolitis (or inflammatory bowel disease) due to chronic biological activity (Sculler eral., 1989). The human TNFe is acidic with a p11 of5,3
inflammation via continuous overproduction ofcytokines such as TNFrs and (Aggarwal of ci., 1985). Each INFe subunit consists of an anti parallel B-sandwich and it
30 IFN-gamma(Thl response). 30 participates in a truerformation by an edge-to-thee packing ofIl-sheets. The structure of
the TNFa timer resembles the "jelly-roll" structural motif characteristic ofviral coat

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18

RANTES has also been implicated in atherosclerosis and possibly in
postangioplasty restenosis(Schall, 1991). The participation ofMCP-1 in atherosclerosis has been studied to a greater extent Recently reRNAs for RANTES. MIP-1 a and
M]P-113 have been detected MkAW in normal carotid plaque and heart transplant
5 atherosclerosis. RANTES mRNA is not detected in the same cells expressing MIP-la and
MW-Ill, but it is expressed in lymphocytes and macrophages typically more proximal to
the lumen. The data argue forpositive feed-back mechanism for the CC cheniokines and possible differential expression ofthese chemokines at variousstagesin the progression of arterial disease-
10 Finally, elevated RANTES levels have been correlated with endometriosis (Kborram etid., 1993). RANTES levels were elevated in pelvic fluids from women with endometsiosis, and these levels correlate with the severity ofthe disease.
Protein Homology between HumenaiidAnimaf The marine RANTES has been
cloned (Schaff et at., 1992). Sequence analysis revealed 85% amino acid identity between
15 the human and mouse proteins. The human and muthieRANlES exhibit immune
crossrdactivity. Boyden chamber chemotaxis.experirnents reveal some lkofspecies specificity in monocyte chemoanractant potential, as recombinantmURANTES attracts human monocytes in a dose-dependentfirshion in Who. Also, hRANTES transfection into mouse tumor cell lines produce tumorsIn which the secretion ofhRANTES by those
20 tumors correlates with increased nimmnt monocyte infiltration in vivo (Seball et aL, l992)

SELEX

TiAtnethod for the in Wmevolution ofnucleic acid molecules with highly specific binding to target molecules has been developed. Thismethod, Systematic Evolution of
25 Ligands by EXponential enrichment, termed SELEX, is described in United States Patent
Application Serial No. 07/536,428. entitled "Systematic Evolution ofLigandsb ExpouentialEnriclrrnerrt." now abandoned, United States Patent Application Serial No. 07/714.131, filed June 10,1991. entitled "Nucleic Acid Uganda,’ United States Patent Application Serial No. 07/931,473, tiled August 17,1992, entitled "Nucleic Acid
30 Ligands," now United States Patent No. 5,270,163 (see also PCfJUS9I/04078). each of
Which is herein specifically incorporated by reference. Each ofthese applications.

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collectively referred to herein as the SELEX Patent Applications, describes a flnidarnentally novel method formaking anucleic acid ligand to any desired target molecule.
The SELEX method involves selection from amreofcandidate---,
5 oligonucleolides an4cp-wise iterations ofbinding, partitioning and ampljflcation
thttiaihajeneral selectron scheme to achieve virtually any desired criterion ofbut

segment of omired seqpence,jbe SELEXmethodiricludes, ep ofcontactingthe--. thargetunder ons favorable fbnbindmg paranonmgunbound.

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cyc1csas.de eld fic nucleic acidilgands to the
15 targetmolecule-
The basic SELEX method has been modified to achieve a number ofspecific
objectives. For example, United States Patent Application Serial No. 07/960,093. filed October 14,1992 entitled "Method for Selecting Nucleic Acids on the Basis ofStructure," describes the use ofSELEX in conjunction with gel electrophoresisto select nucleic acid
20 molecules with specific structural characteristics,such as bent DNA- United States Patent
Application Serial No. 081123,935, filed September 17, 1993. entitled "Photoselection of Nucleic Acid Ligands" describes a SELEX based method forselecting nucleic acid ligands containing photoreactive groups capable ofbinding andlor photocrosslinking to and/or photoinactivating a target molecule United States Patent Application Serial No.
25 08/134,028, filed October 7, 1993, entitled "High-Affinity Nucleic Acid Ligands That
Discriminate Between Theophylline and Caffeine," describes amethod for identifying
highly specific nucleic acid ligands able to discriminate between closely related molecules, termed Counter-SELEX. United States Patent Application Serial No. 081143,564, filed October 25, 1993, entitled "Systematic Evolution ofLigards by EXponential Enrichment:
30 Solution SELEX," describes a SELEX-based method which achieves highly efficient
partitioning between oligonucleotides having high and low affinity for a target molecule.

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United States Patent Application Serial No. 07/964,624, filed October 21, 1992, entitled ’Methods ofProducing Nucleic Acid Ligands- describes methodsfor obtaining improved nucleic acid ligands after SELEX has been performed. United States Patent Application Serial No. 081400,440, filed March 8, 1995, entitled "Systematic.Evolution ofLigands by
5 EXponential Enrichment Cherni-SELEX’ describes methods for covalently linking a
ligand to its target
The SELEX method encompassesthe identification ofhigh-affinity nucleic acid ligandErcomagag ffiodified nucleotides conferring unproved characterisbcsonhgand, nikiimed in vivo stability or improved delivery characteniisEllatp1es-ofsuch--
10 mod1ficaions1nc1udechentstitufihmattherlboseandforpiahatnatid/ori,ase-
pes1tton&SELEX identified nucleic acid ligands containing modifidditticleotides are

1993, entitled "High Affinity Nucleic Acid Ligands Containing Modified Nucleotides,"
that describes oigonucleotides containing nucleotide derivatives chemically modified at
15 the 5-and 7-positions ofpyrirnidines. United States Patent Application Serial No.
08/134,028, supra describes highly specific nucleic acid ligands containing one ormore nucleotides modified with 2’-amino (2.NH2), 2-flnóxo (2’-P), and/or T-0-methyl
(2’-OMe). United States Patent Application Serial No.08/264,029, filed June 22,1994, entitled "Novel Method ofPreparation of2’ Modified Pyrirnidine Intramolecular
20 Nucleophilic Displacement,’ describes oligonucleotides containing various 2’-modified
pyrimidines.
The SELEX method encompasses combining selected oligonucleotides with other
selected oligonucleotides and non-oligonucleotide functional units as described in United States Patent Application Serial No. 0804,063, filed August 2, 1994. entitled
25 ’Systematic Evolution ofLigands by Exponential Enrichment Chimeric SELEX" and
United States Patent Application Serial No. 08/234,997, filed April 28, 1994, entitled ’Systematic Evolution ofLigands by Exponential Enrichment Blended SELEX,’ respectively. These applications allow the combination ofthe broad array ofshapes and other properties, and the efficient amplification and replication properties, of
30 oligonucleotides with the desirable properties ofother molecules. Each ofthe above

WO 96148717 PCJUS964W537
21

described patent applications which describe modifications ofthe basic SELEX procedure are specifically incorporated by reference herein in their entirety.

5 The present invention includes methods ofidentifying and producing nucleic acid ligandsto cytokines and the nucleic acid ligands so Identified and produced. In particular, RNA sequences are provided that are capable ofbinding specifically to WN-gamma, IL4, EL-10, and ThFa. In addition, DNA sequences are provided that are capable ofbinding specificallyto RANTES. Specifically included in the invention are the RNA ligand.
10 sequences shown inTables 3,4,7,8, 10, and 12 (SEQ. ID NOS:7-73; 79-185; 189-205;
209-255).
Euncleded-in-thisinveniorisfncftHigands
and nucleic acid sequences gthninga cªadidtitd niaitumofnucimc acids, (b) con_ctingtbe candidatemuttureofnucleic acids
15 withca eytol (c) partitioning betweenmembers ofsaid candidate mixture on
dfflMiti tIlTe cytokine axid(4 hfysngth selectedrn sythld amixture-of -nudeic acidsóÆcbedfor nucleic acid seqpences with affinity for binding to the cytokine.
Further included in this invention isamethod ofidentifying nucleic acid ligands
20 and nucleic acid ligand sequencesto a cytokine selected from the group consisting of
IFN-gamma, IL-4, IL-b, TNFn, and RANTES comprising the steps of(a) preparing a candidate mixture ofnucleic acids, (b) contacting the candidate mixture ofnucleic acids
with said cytokine, (c) partitioning between members ofsaid candidate mixture on the
basis ofaffinity to said cytokine, and (d) amplifying the selected molecules to yield a
25 mixture ofnucleic acids enriched for nucleic acid sequences with a relatively higher
affinity for binding to said cytokine.
Marc specifically, the present invention includesthe RNA ligandsto IFN-gamma,
IL4, IL-b, and NFn identified according to the above-described method, including
those ligands shown in Tables 3,4,7,8,10, and 12 (SEQ ID NOS-.7-73; 79-185; 189-205;
30 209-255). Also included are RNA Ligands to IFN-gamma, ll..4, IL-b, and TNFn that are
substantially homologous to any ofthe given ligands and that have substantially the same

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ability to bind IPN-gamnra, IL4, IL-10, and TNFa and inhibit the function of 2) The candidate mixture is contacted with the selected target wider conditions
WN-gamma, IL-4, IL-id. and TNF.z. Further included in this invention are nucleic acid favorable for binding between the target and members ofthe candidate mixture Under
ligands to EM-garnnra, IL-4, IL-ID, and ThFu that have substantially the saute struaj these circumstances, the interaction between the target and the nucleic acids ofthe
form as the ligands presented herein and that have substantially the same ability to bind candidate mixture can be considered as forming nucleic acid-target pairs between the
5 WN-ganuna LL-4, IL-ID, and TNFr and inhibit the function ofIFN-gamma, IL-4 IL-10, 5 target and those nucleic acids having the strongest affinity for the target
andINFu 3) The nucleic acids with the highest affinity for the target are partitioned from
The present invention also includes modified nucleotide sequences based on the those nucleic acids with lesser affinity to the target Because only an extremely small
nucleic acid ligands identified herein and mixtures ofthe seine, number ofsequences (and possibly only one molecule ofnucleic acid) corresponding to
the highest affinity nucleic acids exist in the candidate mixture, it is generally desirable to
10 DMLED RIPTION OF THEINVENTION 10 set the partitioning criteria so that a significant amount ofthe nucleic acids in the
This application describes high-affinity nucleic acid ligandsto cytokines identified candidate mixture (approximately 5-500%) are retained during partitioning.
through the method known as SELEX. SELEX is described in U.S. Patent Application 4) Those nucleic acids selected during partitioning as having the relatively higher
Serial No. 07/536,428, entitled Systematic Evolution ofLigands by EXponential affinity to the target are then amplified to create anewcandidate mixture that is enriched
Enrichment now abandoned, U.S. Patent Application Serial No. 07/714,131, filed June in nucleic acids having a relatively higher affinity for the target
15 10, 1991, entitled Nucleic Acid Ligands, United States Patent Application Serial No. 5) By repeating the partitioning and amplifying steps above, the newly formed
07/931,473, filed August!?, 1992, entitled Nucleic Acid Ligands, now United States candidate mixture contains fewer and fewer weakly binding sequences, and the average
PatentNo. 5,270,163, (see attn PCTIIJS9I/04078). These appijeations, each speciflcfly degree of affinity ofthe nucleic acidsto the target will generally increase. Taken to its
incorporated herein by reference, are collectively called the SELEX Patent Applications. extreme, the SELEX process will yield a candidate mixture containing one or a small
In its most basic form, the SELEX process may be defined by the following series number ofunique nucleic acids representing those nucleic acids from the original
20 ofsteps: 20 candidate mixture basing the highest affinity to the target molecule.
I) A candidate mixture ofnucLeic acids ofdifibring sequence is prepared. The The SELEX Patent Applications describe and elaborate on this process in great
candidate mixture generally includes regions offixed sequences (in, each ofthe members detail. Included are targets that can be used in the process; methods for partitioning
ofthe candidate mixture contains the same sequences in the same location) and regions of nucleic acids within a candidate mixture; and methods for amplifying partitioned nucleic
randomized sequences. The fixed sequence regions are selected either (a) to assist in the acids to generate enriched candidate mixture. The SELEX Patent Applications also
25 amplification steps described below, (b) to mimic a sequence known to bind to the target, 25 describe ligands obtained to a number oftarget species, including both protein targets
or (c) to enhance the concentration ofa given structural arrangement ofthe nucleic acids in where the protein is and is not a nucleic acid binding protein.
the candidate mixture. The randomized sequences can be totally randomized (i.e., the The nucleic acid ligands described herein can be coruplexed with a lipophilic
probability offinding a base at any position being one in four) or only partially compound (e.g., cholesterol) or attached to or encapsulated in a complex comprised of
randomized (e.g., the probability offinding a base at any location can be selected at any lipophilic components (e.g., aliposorne). The compierced nucleic acid ligands can enhance
30 level between Oand 100 petuent). 30 the cellular uptake ofthe nucleic acid ligands by a cell for delivery ofthe nucleic acid
ligands to an intracellular target. U.S. Patent Application No. 08/434.465, filed May 4,

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1995, entitled "Nucleic Acid Ligand Complexes," which is incorporated in its entirety effect; (3) be as stable as possible; and (4) be a specific ligand to the chosen target In
herein, describes a for preparing a therapeutic or diagnostic complex comprised of mostsituations, it is preferred that the nucleic acid ligand have the highest possible affinity
a nucleic acid ligand and a lipophilic compound or a non-immunogenic, high molecular to the target.
weight compound. In co-pending and commonly assigned U.S. Patent Application Serial No.
S The methods described herein and the nucleic acid ligands identified by such 5 07/964,624, filed October 21, 1992 624), methods are described for obtaining improved
methods ate useful for both therapeutic and diagnostic purposes. Therapeutic uses include nucleic acid ligands after SELEX has been performed. The ’624 application, entitled
the treatment or prevention ofdiseases or medical conditions in human patients. Methods ofProducmg Nucleic Acid Uganda, is specifically ncorporated herein by
Diagnostic utilization may include both in viva or in vitro diagnostic applications. The reference.
SELEX method generally, and the specific adaptations ofthe SELEX method taught and This invention Includesthe SELEX process for identification ofnucleic acid
10 claimed herein specifically, are particularly suited for diagnostic applications. SELEX 10 ligands ofcytokines. Cytokines are a diverse group ofsmall proteinsthat mediate cell
identifies nucleic acid ligandsthat are able to bind targets with high affinity and with signaling/communication. Cytokines include immune! hematopoletins (e.g.,EPO,
surprising specificity. These characteristics are, of course, the desired properties one GM-CSF, G-CSF, LW, OSM CNTF GK PRL, IL-2, IL-3, IL-4, IL-5, I1-6, IL-7, I1-9,
skilled in the art would seek in a diagnostic ligand. IL-b, IL-12), interferons (e.g.)FNa, IFND, IFN-gamma), ThF-related molecules
The nucleic acid ligands ofthe present invention may be routinely adapted for (eg.,ThFe. IFN, gpn (CD40-L), CD27-L, CD30-L,NGF), and chemokines (e.g, PF4,
diagnostic purposes according to any number oftechniques employed by those skilled in PBP, gina, MIG, ENA-78, MIPIa, MIPIII, MCP-1, 1.309, HCI4, CIO, RANTES,
the art Diagnostic agents need only be able to allow the userto identify the presence of a. Mll’-l). In one embodiment, cytokines are derived from I-lymphocytes.
given target at a particular locale or concentration. Simply the ability to form binding In the present invention, SELEX experiments were performed in olderto identify
pairs with the target may be sufficient to trigger a positive signal for diagnostic purposes. RNA with specific high affinity forthe cytokines JFN-gamma, IL.4, IL-10, bTNFa, and
Those skilled in the art would also be able to adapt any nucleic acid ligand by procedures RANTES from degenerate libraries containing 30 or 40 random positions (40N for
20 known in the art to incorporate a labeling tag in order track the presence ofsuch ligand. 20 [FM-gamma, IL-4, H,10 and RANTES; 30N for bTNFe) (Tables 1,5,9,11, and 16).
Such a tag could be used in a number ofdiagnostic procedures. The nucleic acid ligands This invention includes the specific lilA ligands to IFN-gamma, 11-4, IL-ID, and We
to cytokines described herein may specifically be used for identification ofthe cytokine shown in Tables 3,4,7,8, 10, and 12 (SEQ ID NOS:7-73; 79-185; 189-205; 209-255),
proteins. identified by the methods described in Examples 1,3,5,7, and 12. This invention further
SELEX provides high affinity ligands ofatarget molecule. This represents a includes RNA ligands to IFN-gamma, IL-4, IL-JO, and ThFa which inhibit the function of
25 singular achievement that is unprecedented in the field ofnucleic acids research. The 25 IFN-gamma, IL-4, IL-JO, and TNFa. This invention further includes DNA ligandsto
present invention applies the SELEX procedure to the specific target In the Example which inhibit the function ofRANTES- The scope ofthe ligands covered by
section below, the experimental parameters used to isolate and identify the nucleic acid this invention extendsto all nucleic acid ligands ofIFN-gamma, IL-4, IL-b, TNF a, and
uganda to cytokines are described. RANTES modified and unmodified, identified according to the SELEX procedure. More
In order to produce nucleic acids desirable for use as a pharmaceutical, it is specifically, this invention includes nucleic acid sequencesthat are substantially
30 preferred that the nucleic acid ligand ())binds to the target in a manner capable of 30 homologousto the ligands shown in Tables 3,4.7, 9, 10, and 12 (SEQ ID NOS:7-73;
achieving desired effect on the target, (2) be assmall as possible to obtain the desired 79-185; 189-205; 209-255). By substantially homologous it is meant a degree ofprimary

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squenhomology ce In excess of70%, mostprelhmbly wexcme of80% A rłttize

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eqUcflce homologies ofthe hgands ofIFN-gamma, 11,4 IL 10 and ThFa shown in
Tables3 4 7 S. 10 and 12(SEQIDNOS7-73 79 185 189-205 209255)shcwsihat
sequences With little or no prunary homolqW May have mibstantially thD sarne ability to

I A method ofideutiiong nucleic acid ligandsto a cytokine, comprising
-5: bid IFN-ganima, IL-4, IL-b, andThFs. For thew reasons, this.inverition also includes- a) contacting a candidate mixture ofnucleic acids with said.cytokine,
nucleic acid ligands that have substantially the same structure and ability to bind wherein nucleic acids having an increased affinity to said cytokine relative to the candidate
IPN-gainma, 11.4, IL-b, and ThFn asthe nucleic said uganda shown in Tables 34 may be partitioned fromthe remWnder ofthe candidate mixture; and
10 and 12 (SEQ ID NOS 7-73 79-185 189-205 209-255) Substantially the seine ability b) partitioning the increased affinity nucleic acids from the remainder of
to bind amma, ii. and TNFa.meansthat the affinity is willhin one at two

the candidate nuixtrn’e; and

10 orders ofmagnitude ofthe affinity ofthe bgands described hareim It iswell within the c) amplifying the increased affinity nucleic acidsto yield amixture of
skill ofthose ofordinary skill in the art to determine whetheragiven sequence

nucleic acids enriched fornucleic acid sequences with relatively bigheT affinity and
substantially hiamolopusto those specifically dawlWherein hassubstatially the specificity for binding to said cytokme, whereby nucleic acid ligands ofsaid cytokum may
same ability to bind ilN.gntnm, IL-4,M-10, and TIIFe be identified.
This invention also includesthe hgandsas described 2 The method ofclaim I further coinpusini
-re made in orderto increase dw in vftstubftafthe-figandor d) repeating steps 4, bj and c)
toenhance ormediate the deliveiy ofthe hgand. Examples ofsuch modifications include 3 Themethod ofclaim I wherein said candidate mixture ofnucleic acids is
chemicalsubstitutions at the sugar and! orphosphate and/or base positions ofa given comptised ofsingle stranded nucleic acids.
nucleic acid sequence. See, e.g, U.S.Patent Application Serial No 08/117991 filed 4 The method ofclaun3 wherein said single stranded nucleic acids are
September 9 1993 entitled High Aflinjty Nucleic Acid LigandsQmtamrng Modified ribonticleic acids.
20 Nucleotides which is specicaiiy mcaqioxaicrt itereinby rd=nm Othamodifications S The method ofclaim4 aheinnisaid nucleic acids aremodified nucleic muxk
are known to one ofordinary skill inthesit Such mo.canousmay be _’ 6 Themethod ofclaim 5 wherein said nucleic acids are 2-ammo (2-NH2)
post SELEX (modification ofpreviously identified unmodified hgauds) modified ribonucleic acids.
incorporation into the process 7 The method ofclaim 5 wherein said nucleic acids are 2’ fluoro (2’-F) modified
As described above, because oftheir ability to selectively bind IFN-gamma, IL.4 nbonucleic acids.
25 IL 10 hThFe and RANTES the nucleic acid bgundsto IFN-gamma, IL-4 11.10 irn S The method ofclaim 3 wherein said single stranded nucleic acids are
and RANTES described harem are useful as pharmaceuticals. Thisinvention, therefore, deoxyribonucleic acids.
also includes a methodofinhibiting cytokrne function byadministration ofa nucleic acid 9 The method ofclaim I wherein said cYtid.me Is
ligand capable ofbinding to a cytokine selected from the group consisting ofIFN-gamma, IL-10,IL-4 TNF-alpha. and RANTES
Therapeutic compositions ofthe nucleic *64hgands may behnimsrered W. The method ofcLaim 1 wherein said cyzokute is IFN-gamma.
o parenxeraUy by ugeclion1 although other effective forms, such 11 The method ofclaim I wherein said cytokme isIL-lU
intraarticulariijection. lrj orally ji’ 12. The method ofolaim I wherein said ytokine a 1L-4

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13. The method ofelaim I wherein said cytokine is ThF-alpb& aiontacting a candida_niature elm cleic acids with.said.cytokizie,
14 The method ofclaim I wherein said cytokme isRAN1S wherein1WclaiCcandidate
15. A method for treatingacytok ne-mediated disease comprising administering a _mbaure ypaone&fromiha.remin thecandidata.muxtomank
pharmaceutically effective amount ofa nucleic acid liganilofacytokine. theffmainderof_
16. The method ofclaim 15 wherein said nucleic acid ligand ofacytokine is the cazAidaft
identified according to the method ofclaim! c)ainpyipg the increased affinitynuelciecrdstqyxekl a.mixture of
17 The method ofclaim 16 wherein said cytokme is JFN gamma. nuc1efrmue1aimid
IS. The method ofclaim I7 wherein said ligand is selected from one ofthe ligands
ofTables 3 and 4(SEQ ID N08:7-73). bejentified..,,.
19. Themethod ofclaim 16 wherein said cytokine is 11,4. 30. The purified and isolated non-naturally occurring ribosaicleic acid ligand of
20. The method ofclaim!? wherein said ligand is selected from one ofthe ligands claim 27, wherein said ligand isIPN-gammai
ofTables? and (SEQ JDNOS:100-185). 31. The purified and isolated non-naturally occurring ribonucleic acid ligand to
21. The method ofclaim 16 said cytokinc is fl40 LfN..ganuna ofclaim 30 wherein said ligand is selected from the group consisting ofthe
22. The method ofclaim 21 wherein said ligand is selected from one ofthe ligands sequences set forth in Tables 3 and 4(SEQ R)N08:7-73).
ofTable 10 (SEQ ID NOS:189-204 32. The purified and isolated non-naturally occurring ribonueleic acid Iigandto
23. The method ofclaire 16 wherein said cytokine isNP-alpha. IFN-gamma ofclaim 30 wherein said ligandis substantially homologousto and has
24. The method ofclaim 23 wherein said ligand isselected fromone of the ligands substantially the some ability to bind MN-gamma as a ligand selected from the group
ofTable 12 (SEQ IDNQS.209-255). consisting ofthe sequences set forth inTables 3 and 4 (SEQ IDNOS:7-73).
25. Apurifiedand-i 33. Tim purified and isolated non-naturally occurring ribomicleic acid ligand to
IFN-gamma ofclaim 30 wherein said ligand has substantially the some structure and
2&Thepkdisobsenou-lIyoccingnuc1eic-acidiigand-ofcIaim substantially the same ability to bind WN-gamma as aligand selected from the group
25 wherein said nucleic acidligand issingle-stranded..-.., consisting ofthe sequences set forth in Tables 3 and (SEQ I13NOS;7-73).
27 Thepinfle&and isolated non-nati fly ccumng nucleic acid ligand ofclaim 34 Ile punfledand isolated non naturally occurring nbnucleic add ligand of
claim 27 wherein said ligand is to I1.4.
28. Thedati&isclateduron=natmnIty-occing-nuc1eicacid.ligan&ofclaim-. 35. The purified and isolated non-naturally occurring ribonucleic acid ligand to IL-
26 wherein sazdnuclmcaci&ligdeoxynbonucleic-acr&. 4 ofclaim 34 wherein said ligand isselected from the group consisting ofthe sequences
29knucicicacid-ligantoy4cuti0ataccordizgteilaziheth6d set forth inTables? and 8 (SEQ ID NOS:79-1 85).
- Comprising_’-,36.The Purified and isolated non-naturally occuningiibonucleic acid ligand to IL-4 ofclaim 34 whereto said ligand is substantially homologous to and has substantially the

96/40717 PCrAYS96AMS37 WO96(40717

170 171

same ability to bind IL-4 as a ligand selected from the group consisting ofthe sequences substantially the same ability to bind ThF-alpha as a ligand selected from the group
set loath in Tablesland 8 (SEQ ID NOS:79-1 85). consisting ofthe sequences set forth in Table 12 (SEQ ID NOS: 189-.205).
37. The purified and isolated non-naturally occurring ribonucleic acid ligand to j 46. The purified and isolated non-naturally occurring nucleic acid ligand ofclaim
4 of claim 34 wherein said ligand has substantially the same structure and substantially the 7 25 wherein said ligand is to RANThS.
same ability to bind IL-4 as a ligand selected from the group consisting ofthe sequences
set forth in Tables land 8 (SEQ II) NUS:79-185).
38. The purified and isolated non-naturally occurring ribomimeic acid ligand of
claim 27 wherein said ligand is to IL-b.
39. The purified and isolated non-naturally occurring ribonucleic acid ligand to IL-
lllofelaim 3* wherein said ligand is selected from the group consisting ofthe sequences set forth in Table 10 (SEQ ID NOS:189-205).
40. The purified and isolated non-naturally occurring ribonucleic acid Ligand to 11,
10 olclaim 38 wherein said ligand is substantially homologous to and has substantially the same ability to bind IL-I0 asligand selected from the group consisting ofthe sequences set foreth inTable 10 (SEQ Ii) 1405:189-205).
41. The purified and isolated non-naturally occurring ribonucleic acid ligand to IL-
10 ofclaim 38 whenibisaid ligand has substantially the some structure and substantially the sameability to bind IL-lO as a ligand selected from the group consisting ofthe sequences set fosthiuTable 10 (SEQ IDNOS:l*9-205).
42. The purified and isolated non-naturally occurring ribonucleic acid ligand of
claim 27 wherein said ligand is to TNF-alpha.
43. The purified and isolated non-naturally occurring ribonucleic acid ligand to TNF-alpha ofclaim 42 wherein said ligand is selected from the group consisting ofthe sequences set forth in Table 12 (SEQ ID N0Si09-255).
44. The purified and isolated non-naturally occurring ribonucleic acid ligand to TNF-alpha ofclaim 42 wherein said ligand is substantially homologous to and has substantially the same ability to bind TNF-alpha as a ligand selected the group consisting ofthe sequences set forth in Table 12 (SEQ IDNO&2O9-255).
45. The purified and isolated non-naturally o=aring ribonucleic acid ligand to TNF-alpha ofclaim 42 wherein said ligand has substantially the same structure and

SUPERNTENDENCIA DE INDUSTRIA V COMERCIO DIRECCION DE NUEVAS CREACIONES

2020.

Bogot& D.C.,

Abogado DILIA RODR1GUEZ DALEMAN

ASUNTO Radioacióæ 06-100210
TrÆmite 011
Evento 378
Aetuaciôn 519

Oficlo &19440
Folios 016

Paterite de lnvenciôn fl Patente de Modefo de Utilidad
Via Nacional

Via PCT (fase nacional) L -_J Cap.f F Cap. II

No. Solicitud Internacional PCT/US20051007666
Fecha de Presentaciön Internacional 07 Marzo 2005

FOR

1.

Como resultado del Examen de Fondo reallzado, con fundamento en el ArtIculo 45 de Ia DecisiOn 486 de la ComisiOn de la Comunidad Andina, se comunica:

1. Documentos estudiados

DescripciOn:
Reivindicaciones:
Dib Uj Os:
Prioridad:

2. Objeto de la invención

Folios 324 a 558
Folios 559 a 573
Folios 575 a 597
US 60.550.962
US 60.608.046

04 Octubre 2006
04 Octubre 2006
04 Octubre 2006
05 Marzo 2004

07 Septiembre 2004

Titulo de la solicitud: "Aptâmeros para la famiUa citoquina IL-12 humana y su uso como terapØuticos para enfermedades autoinmunes".

El problema planteado en esta solicitud consiste en la necesidad de agentes para modular, estimuiar o inhibir la IL-23 o la IL-12 las cuales intervienen en los procesos de enfØrmedades autoinmunes y cancerIgenas.

Para resolver este problema tØcnico la solicitud en estudlo proporciona ligadores de ÆcidosnuclØicos tambiØn Ifamados aptâmeros los cuales Se unen especificamente al IL-23 para modular, estimular o inhibir dicha citoquina.

El objeto de la presente Solicitud se relaciona con la ClasificaciOn Internacional de Patentes (CIP): A61K 39/395

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

El objeto de]as reivindiôaciones 59 a 65, 67 a 72 que corresponde a un mØtodo de tratamiento, no es patentable de acuerdo con el Art citado.

Primer Ar-ticulo 45- Expediente 06-100210

4. De la Soilcitud de Patente

 Reivindicaciones (Art 30 D 486, Art 22 PCT)

Las reivindicaciones I a 3, 46 a 48 no son claras pues no caracterizan adecuadamente el aptÆmeroreclamado. El aptÆmeroreclamado es caracterizado par su funciOn a acción biológica y no por su secuencia, estructura, etc. Asimismo se definen variantes quo tampoco se encuentran caracterizadas por su secuencia. Es importante mencionar que los aptÆmeros son oligonucleotidos de cadena sencilla tienen una region central de tamaæo variable y con una secuencia aleatoria, y dos regiones flanqueantes do secuencia conocida, las cuales deben caracterizarse en su totalidad mediante su secuencia. Par otro lado se debe dØfinir si el aptÆmero es detipo ADN, ARN 0 hibridos ADN/ARN.

Las reivindicaciones 29 a 36 son las que definen do mejor manera el objeto de la presente solicitud. Sin embargo) so sugiere establecer cual de los posibles grupos de secuencias corresponden al aptªmero que se pretende proteger, ya quo las secuencias presentan diferencias significativas en el ntmero y tipo do nucleótid.os presentes y no a todas ellas se les atribuye la funciOn biolOgica rectamada. Do otro lado so debe caracterizar el aptÆmeroteniendo en cuenta tambiØn las modificaciones quimicas realizadas a este.

Las reivindicaciones 4 a 6, 49 a 50 y 56 a 57 caracterizan el aptÆmero porla estructura a la cual se une (ya sea IL-23 0 IL-12) y no par su estructura o secuencias quo permiten la union a estas. Asimismo dichas citoquinas (1L23 IL-
12) son caracterizadas por. un porcentaje de identidad de las secuencias de aminoÆcidos que las conforman to cual Ileva a quo se incluyan un nümero indeterminado do secuencias quo podrian unirse con el aptªmero objeto do la presente invenciOn.

Las expresiones "de menos do", "esencialmente", "substancialmente", "at menos" son imprecisas, par 10 cual no permiten distinguir la invenciOn del estado de la tØcnica. Se sugiere sustituirlas par un tØrmino preciso o un rango concreto.

Las reivindicaciones 9 y 10 contienen los tØrminos "single stranded" y deoxyacido" los cuales corresponden a palabras en ingles y no permiten dar claridad at capitulo reivindicatorio. Se sugiere modificar dichos tØrminos par su correspondiente en espanol.

Las reivindicaciones 12 a 15 caracterizan el aptâmero par un parªmetro (constante do disociaciOn). Debido a que el aptÆmero puede definirse par su secuencia especifica, se sugiere definirlo por sus caracteristicas esenciales, estructurales o funcionales.

- La reivindicaciôn 16 tiene una falsa dependencia con la reivindicaciOn 7, ya quo no puede establecerse a que estructura le corresponde la modificaciOn qulmica reetamada

- Las reivindicaciones 16 a 18, 21 a 24, 37 a 39 y 42 a 45 que so refieren a una modificaciOn qulmica realizada at aptÆmero reclamado, noson concisas puesto que no definen inequivocamente quo tipo de modificaciOn, el nümero do modificaciones, la naturaleza, estructura y posiciOn de estas. Se sugiere definir y

Primer Articulo 45- ExDediente 06-100210

timitar el tipo de modificaciOri sobre que estructura o secuencia genera, la
naturaleza. etc,

- Las reivindicaciôn 29 y 30 reclaman et mismo objeto do invención portal razón so
sugiere at solicitante suprimir alguna do las dos

Las reivindicaciones, 31 y 32 caracterizan el aptªmero por un porcentaje de identidad de las secuencias que to conforman. La anterior no es correcto pues no define inequlvocamerite la secuencia objeto de la invenciOn y los porcentajes de identidad do 80% y 90% incluyen un nümero indeterminado de secuencias quo podrIan no tener relaciOn con el epitope at cuat va dirigido et objeto de la presente invención. Se sugiere modificar las reivindicaciones de tat manera quo so caractericen con una secuencia a secuencias concretas

Las reivindicaciones 25 a 28, 52 a 55 se refieren a funciones de modulación, inhibiciOn, etc., del aptÆmerorectamado, to cuat corresponde a un resuttado a alcanzar puesto quo la reivindicaciôn inctuiria no solo la sotuciOn propuesta par el solicitante, sino todas las alternativas presentes a futuras quo Ileguen a ese resultado. Se sugiere reemplazarlo par las caracteristicas esenciales, estructurales a funcionates, que contribuyen a la obtenciOn de ese efecto,

- La reMndicaciOn 58 contiene la expresiOn "cantidad terapØuticamente efectiva"
que Os imprecisa, par to cuat no permite distinguir la invenciOn del estado de la tØcnica y no limita el alcance de la reivindicaciOn. Se sugiere sustituirta por un tØrmino precise o un rango concrete.

Las reivindicaciones 73 a 75 Indican et use que puede darse at aptÆmero reivindicado, el cual no es una caracteristica tØcnica que defina a la invenciOn. Do acuerdo con to divulgado en el capitulo descriptive las secuencias de aptÆmeros probados quo presentan un efecto diferenciat en cuanto at su estabilidad son las
identificadas coma ID SEQ 307 a 309 y 312 a 314. Par tal razOn, so sugiere at solicitante restringir et objeto de invenciOn a las realizaciones preferidas de acuerdo con to divulgado en el capitulo descriptivo.

Descripción (Art 28 D 486)

- Dade quo esta solicitud se refiere a secuencias do nucleôtidos a aminoÆcidos, deben presentarse las listas en forma separada de la descripcion, baja et tituto:

"Listado do secuencias" y anexarlas en formato digital.

S. Determinación del Estado de la TØcnica

El Estado de la TØcnica se determinô con base en la fecha de presentaciôn de la solicitud do prioridad (05 Marzo de 2004) y se encontraron los siguientes documentos quo anticipan et objeto de esta solicitud:

Item Documento TtuIo Fecha de __________________________________ Publicacion

Nucleic acid Iigands which bind to Hepatocite Dl US 6344321 Drown Factor/Scattwer Factor (HGF/SF) or its 05 Febrero 2002 ____ _____ recetor C-met
rD2......c. 9071 Iii’.!’!f!YJ!cacldl!dsofcytokinesL1g Diciembre

Primer Articulo 45- Expedierite 06-100210

Dl (folios 630 a 633) divulga ligandos de Æcido nucleico del factor do crecimiento do hepatocitos (HGF) y su receptor c-met. Los ligandos de âcido nucleico do Ia invenciôn son aislados por el mØtodo SELEX. Los ligandos do Æcido nucleico do la invenciôn son Utiles come agentes do diagnOstico y agentes terapØuticos para enfermedades relacionadas con
HGF.

URL:http:f/worldwideespacenet.corn/publicatIonDetails/bibliO?DBEPODOC&l l0&adjacenttr ue&Iocaleen_gb&FTD&datØ20020205&CCUS&NR634432181 &KCB1

D2 (folios 634 a 639) divulga mØtodos para la identificaciôn y preparaciOn de ligandos de
â cido nuclØico do alta afinidad a las citoquinas. El mØtodo utilizado se denomina SELEX.. Esta invención incluye especificamente los mØtodos para la identificaciôn do ligandos de alta afinidad do Æcidosnucleicos do las citoquinas IFN-gamma, IL-4, IL-10, TNFa, y RANTES. URL: http://wortdwide.espacenet,com/publiCatiOnDetailS/deSCriPtiOn?CCWO&NR=964O7 1 7A1 &KCA1 &FTD&date 1996121 9&DB=EPODOC&localeen_gb

6. Examen do Patentabilidad (Art 14 D486)

Novedad (AT8 486)

La reivindicación 34 consiste en el aptÆmero dela reivindicaciOn 1, en donde el aptÆmerocomprende 8 nucleOtidos contiguos quo son idØnticos a una secuencia do 8 nucleOtidos contiguos en la region do secuencia Unica de cualquiera do las secuencias seleccionada del grupo que consiste do: SEQ ID Nos 13-66, SEQ ID Nos 13-66, SEQ ID Nos 13-66, SEQ ID Nos 71-88, SEQ ID Nos 91-96, SEQ ID Nos 103-118, SEQ ID Nos 124-134, SEQ ID Nos 135-159, SEQ ID Nos 162, y SEQ ID Nos 164-172, SEQ ID Nos 176-178, SEQ ID Nos 181-196, y SEQ ID Nos 199-314.

ComparaciOn do las caracteristicas tØcnicas esenciales, con las del Estado de la TØcnica:

CaractŁrIsticasesenciales Dl

Comprende 8 nucleÆtidos contiguos quo son
idØnticos a una secuencia de 8 nucleOtidos en Is
regiOn do secuencia tnica do cuslquiera do las 6 nucleOtidos continues
secuencias seleccionada del grupo que consiste

S do: -..-...-.-.----.... SEQ IDI99-314 SEQ ID69

(... UGCGGGCGGG...) (GGGAGGACGAUGCGGGCGGG...)

Las caracteristicas esenciales del aptØmero do la reivindicación 34 habian sido divulgadas previamente en Dl, el cual enseæa la secuencia ID 69 lacual corresponde a un aptØmero especifico para la citoquina (HGF) (folio 633, columna 65). Dicha cadena de nucleótidos contiene 10 nucteOtidos contiguos quo corresponde a UGCGGGCGGG, los cuales son idØnticos a una region do secuencia ünica del grupo do secuencias 199 a 314 do la presente solicitud (ver folios 519, 525, 532 a 537).

En consecuencia, Ia reivindicaciOn 34 no es nueva, segün lo divulgado en el documento Dl.

La reivindicaciOn dependiente 35 no menciona alguna caracteristica quo haga nueva al objeto de (a reivindicaciOn 34, por 10 cual se considera que no es nueva.

Primer Articulo 45- Exediente 06-100210

La r&vindicación I consiste en un aptÆmero quese une especIficamente a IL-23

Cornparaciôn do las caracteristicas tØcnicas esenciales, con las del Estado de la TØcnica:

fttro q IL-23 citoauinas

A que uneathversas

El documento D2 se consideran:1 estado de la tØcnica cercano a la invenciOn definida en la reivindicación I porque divulga ligandos de Æcidonuclelco (RNA) de alta afinidad tambiØn denominados aptÆmerospara citoquinas (folio 634, pagina 2, lInea 4, 5, 10; folio 639, pagina 168, reivindicaciOn 25 a 29). Los aptÆmerosson diseæadospara citoquinas especIficas como son IL-4 e IL-1 0 las cuales actüan sabre esistema inmune interviene en Is senalización de enfermedades autoinmunes y de tipo cancer (folio 634, pagina 5, lines 27 a 30; folio 635, pagina 8, lines 12 a 19; pagina 10, lines 2 a 10).

El mØtodo de identificaciôn y diseæo dedichos ligandos se denomina SELEX el cual incluye los paces de ponerse en contacto una mezcla de oligonucleótidos al azar en condiciones favorables para generar una secuencia enriquecida que presenta interacciôn, especificidad y afinidad con una secuencia blanco perteneciente a la citoquina a la molØcula objetivo IL-4, IL-b, TNF-a (folio 636, pagina 19, lines 4 a 6); dichas secuencias son amplificadas y disociadas adecuadamente en diferentes ciclos pare lograr secuencias especificas con alta afinidad (folio 636, pagina 19, lines 10 a 15).

Asimismo, dicho mØtodo incluye nucleOtidos modificados que confieren caracteristicas mejoradas en el ligando, como la mejora de la estabilidad in viva a mejora de las caractersticas de liberaciôn de este. Ejemplos de tales modificaciones inclüyen sustituciones quimicas en Is ribosa y/o fOsforo y/o posiciones de base (folio 637, pagina 20, Ilnea 7 a 13).

La diferericia entre Is invención, definida en la reivindicaciôn 1 ylos aptÆmeros dados a conocer en D2 es que:1 aptÆmero diseæado esespecifico pare la IL-23.

Per Ic tanto, el Problems TØcnico Objetivo que pretende resolver esta invención se puede formular ad: "cómo utilizer las metodologias de SELEX conocidas en el estado de la tØcnica (titiles para la generaciOn de aptÆmerosque sirven come ligandos de alta afinidad para citoquinas) divulgadas en D2 para obtener una secuencia especifica con afinidad para IL-23)

Sin embargo, Is selecciôn de diferentes oligonucleOtidos funcionales para diferentes tipos de ligandos (citoquinas) es conocido y esta selecciôn involucra diferentes pasos los cuales se pueden utilizar pare obtener candidatos pare todo tipo do citoquinas (folio 637, pagina 21, lines 13 a 18). Los pasos incluyen a) poner en contacto la citoquina de elecciOn con una mezcla do candidates do los Æcidos nucleicos, enel que los Æcidos nucleicos que tienen una mayor afinidada dicha citoquina en relaciOn a Is mezcla do candidates podrÆn serrepartidos entre et resto de Is mezcla de candidatos, b) divisiOn de los Æcidos nucleicosa partir de una mayor afinidad que el resto de Is mezcla do candidatos, y c) la amplificaciOn do los Æcidos nucleicos conuna mayor afinidad pare dar una mezcla de Æcidos

Primer Articulo 45- Expediente 06-100210

nucleicos enriquecida par secuencias con una afinidad relativamente alta y la especificidad de union a dicha citoquina, en el que los ligandas de Æcido nucleico de dicha citoquina puede ser identificados (folio 639, pagina 169, reivindicaciOn 29). Lo anterior es.valido para todo tipo de citoquinas proteinas objetivo ya coriocidas en el estado de la tØcnica tal coma la IL-231.

En consecuencia, la persona del oficio normalmente versada en la materia, estaria motivada a utilizar el mOtodo SELEX divulgado en D2, para obtener un aptÆmero que se una especificamente a una citoquina ya conocida en el.estado de (a tØcnica coma es la IL-23 ilegando asi al objeto de la reivindicaciOn 1 de la solicitud. Por lo cual se considera que no cumple con el requisito de nivel inventive

Puesto que las reivindicaciones dependientes 2 a 28 y 46 a 57 no mencionan caracteristicas inventivas adicionales tampoco se pueden considerar inventivas.

7. ConclusiOn

Los requisites de Patentabilidad de la presente Solicitud estÆnafectados asi:

L Dl f US 6344321 34-35 Novedad
L D2 JWO 9640717 1-6 Nivel lnventh,o

En cumplirninto dl artIculo 45 de la DecisiOn 486 de la ComisiOn de la Comunidad Anthna, se lencljccuje debe dar respuesta dentro del tØrmino de sesenta (60) thas contados a partir de (a fecha de notificaciOn de la presente comunicaciOn. En case de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesiOn, se denegarÆ lapatente.

Notifiquesee! presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capitulo quinto del titulo primero de la Circular Unica No. 10 de 2001

JOSÉ LUIS SALAZAR

Director de Nuevas Creaciones (C)

El anterior requerimiento fue notifIcado por fijaciôn en lista, de fecha

Elaborado per: Ana Delia PinzOn Garcia
Revisado Por: Jacqueline Alfonso
Aprobado per: JosØ Luis Salazar Lopez

1 CORDOBA-ODRIQUEZ R. ET AL. IL-23 and 1L-27: Now Members of the Growing Family of IL-12-Relatod Cytokinas with
Important Implicatiors for Therapeutics vol. 3, 2003, panes 715 723

Primer Articulo 45- Expediente 06-100210