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(54) **Title**: APTAMERS TO β-NGF AND THEIR USE IN TREATING β-NGF MEDIATED DISEASES AND DISORDERS

(57) **Abstract**: The present disclosure relates generally to the field of nucleic acids and, more particularly, to aptamers capable of binding to β-NGF; pharmaceutical compositions comprising such β-NGF aptamers; and methods of making and using the same.

APTAMERS TO β-NGF AND THEIR USE IN TREATING β-NGF MEDIATED DISEASES AND DISORDERS

RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application Serial No. 61/323,145, filed April 12, 2010, which is incorporated herein by reference in its entirety.

[0002] Incorporated by reference herein in its entirety is the Sequence Listing entitled "Sequence_listing_ST25.txt", created April 1, 2011, size of 126 kilobytes.

FIELD OF THE INVENTION

[0003] The present disclosure relates generally to the field of nucleic acids and more particularly to aptamers capable of binding to nerve growth factor, more specifically the β subunit of nerve growth factor ("β-NGF"), and useful as therapeutics for preventing, treating or ameliorating pruritus, pruritic conditions and/or other diseases or conditions in which β-NGF has been implicated. The disclosure further relates to materials and methods for the administration of aptamers capable of binding to β-NGF.

BACKGROUND

[0004] The following description provides a summary of information relevant to the present disclosure and is not an admission that any of the information provided or publications referenced herein is prior art to the present disclosure.

[0005] Severe itching negatively impacts the quality of life of millions of people every day. Severe itching may be associated with various health conditions, including pruritic skin conditions, such as scabies, eczema, xerosis, psoriasis and urticaria, as well as systemic conditions, including chronic hepatic or renal disease and lymphoma. Similarly, pain is a common occurrence, being one of the major reasons for visits to a physician. Pain may be associated with numerous types of injuries or conditions, and failure to treat acute pain may lead to chronic pain issues, as well as immune and metabolic disorders. In addition to reducing the quality of life for the individual suffering from pruritus and/or pain, there is a significant impact

on healthcare budgets, particularly in relation to pruritic skin conditions, as well as, chronic pain disorders. Current efforts to manage or treat pruritus and/or pain are widely recognized as being inadequate.

[0006] Neurophysiological research has confirmed the distinctiveness of itch pathways in comparison with those of pain. The itch sensation is perceived and transmitted by dedicated C neurons which are distinct from the nociceptors that process pain sensation (Schmelz, Neurosci. Biobehav. Rev. doi:10.1016/j.neubiorev.2008.12.004, 2009). The dedicated C neurons then transmit the itch stimulus to a specialized class of dorsal horn neurons projecting to the thalamus (Stander and Schmelz, Eur. J. Pain 10:473, 2006). There is believed to be no special itch receptor on peripheral nerve endings and the specificity of itch C neurons is based on their spinal connections to the itch pathway. Differences are observed in the brain activation patterns between itch and pain, such as an absence of detectable activation of the thalamic and somatosensory cortex of the parietal lobe from itch sensation (Yosipovitch *et al.*, Lancet 361:690, 2003).

[0007] Pain is generally classified as either acute or chronic. Acute pain is commonly a response to tissue damage, characterized as short-lived and resolves as the initial damage heals. Chronic pain is persistent and may have no apparent association with a traumatic event. Pain may further be classified based on the mechanistic origin of the pain and includes nociceptive and non-nociceptive. Nociceptive pain is mediated by specific receptors (nociceptive receptors) that are activated by a specific stimulus (injury, inflammation, chemical, etc). Nociceptive pain may further be classified as somatic or visceral. Somatic pain occurs in tissues such as skin, muscle, joints, bones, or ligaments. Somatic pain is generally sharp and localized. Current treatments include use of opioids and non-steroidal anti-inflammatory drugs (NSAIDS). Visceral pain occurs in internal organs. It is frequently a poorly localized pain and is generally treated with opioids.

[0008] Non-nociceptive pain may be further broken down into neuropathic or sympathetic. Neuropathic pain may arise in the peripheral or central nervous system. Neuropathic pain may be associated with degenerative conditions, inflammation, or infectious diseases. This type of pain results in hypersensitivity (hyperalgesia) and is frequently described as shooting or burning. Treatment options include N-methyl-D-aspartate (NMDA) antagonists,

anti-arrhthymics, anti-convulsants, or anti-depressants. Neuropathic pain is frequently resistant to conventional analgesics. Sympathetic pain arises in the sympathetic nervous system as well as the peripheral and central nervous systems and is generally associated with some type of injury. The site of injury may show increased hypersensitivity and abnormal temperature. Treatment generally involves a multi-drug regimen including sympathetic nerve blocks, vasodilatation, anti-convulsives, anti-arrhthymics, and anti-depressants.

[0009] Routine and prolonged treatment of pain with opioid analgesics is not recommended because of the concern for potential addiction, side effects, tolerance, and dependency on the opioid. Opioid side effects can include nausea, vomiting, constipation, respiratory depression, etc. With many current treatments there exists a lack of efficacy, serious side effects, and inability of drug delivery methods to help in adequate pain control. These issues support the need for better pain control therapeutics.

[0010] Although itch and pain are clearly distinct sensations, there are important interactions between itch and pain. It is well known that itch can be reduced by the painful sensation caused by scratching. Yet, analgesics, such as opioids, by acting to diminish pain sensations, can actually enhance itch sensation. Thus, some therapeutics for pain can exacerbate itch symptoms further supporting the need for better therapeutics with the potential to treat both itch and pain.

[0011] Nerve growth factor (NGF) is one of a family of neurotrophic cytokines or neurotrophins. Neurotrophins play a key role in the development and maintenance of both the peripheral and central nervous system by controlling cell survival, differentiation, and apoptosis. In addition to these nervous system functions, NGF has also been shown to increase the release of histamine, the production of mast cells, and the growth and differentiation of B lymphocytes. NGF has also been shown to modulate the basophilic production of certain lipid mediators. The apoptosis of neutrophils may also be suppressed by NGF. All of these factors suggest a role for NGF in the immune system as well as the nervous system.

[0012] The NGF beta chain (β-NGF) is solely responsible for the nerve growth stimulating activity of NGF. In the cell, β-NGF exists as a dimer and binds to two types of cell surface receptors in neuronal and non-neuronal cells. The tertiary structure of the protein is based on three cystine disulfides and two anti-parallel, β-strands. The amino acid homology of

the human, mouse, and rat proteins are about 90%. β-NGF, like all of the neurotrophins, binds to the p75 cell receptor with nM affinity. β-NGF also binds to one of the tyrosine kinase receptors (Trk) in particular, TrkA, with pM affinity. Reaction with the p75 receptor can induce cell death while binding to TrkA promotes cell survival. β-NGF binding to TrkA leads to phosphorylation of the receptor and internal cellular proteins. β-NGF is internalized by receptor-mediated endocytosis. Trk receptors are found in a wide range of non-neuronal tissues.

[0013] Nerve growth factor (NGF) released from keratinocytes in the skin is one of the major mediators that increase dermal nerve density and affect morphology by, among other things, promoting sprouting of nerve fibers (Schmelz, Neurosci. Biobehav. Rev. doi:10.1016/j.neubiorev.2008.12.004, 2009). Patients with chronic pruritus have been found to exhibit increased intradermal nerve fiber density. Further, NGF has been found to increase sensitivity of peripheral neurons by, among other things, triggering the receptor of NGF, tyrosine kinase TrkA (Stander and Schmelz, Eur. J. Pain 10:473, 2006).

[0014] The importance of NGF in mediating pruritus as well as pain is exhibited in the high concentrations of NGF measured in atopic conditions, which may be symptomized by both pruritus and pain. Patients with atopic dermatitis have greatly increased serum levels of NGF which positively correlate with the severity of the condition. Patients with contact dermatitis have higher local NGF concentrations and patients with prurigo nodularis also exhibit higher NGF levels and TrkA activation levels (Schmelz, Neurosci. Biobehav. Rev. doi:10.1016/j.neubiorev.2008.12.004, 2009).

[0015] The effects of anti-NGF antibodies administered systemically by intraperitoneal injection on symptoms in a mouse model for atopic dermatitis having been studied and results "suggest that anti-NGF antibodies block the effects of NGF on the periphery of the nervous system and suppress epidermal innervations, dermatitis and scratching behavior" (Takano *et al.* J. Pharmacol Sci 99:277:284, 2005). Yet, the study found that anti-NGF antibodies did not alter serum NGF levels, did not decrease the NGF concentration in the skin areas tested, and did not completely suppress scratching behavior. Thus, a need to more completely reduce or eliminate itching associated with atopic dermatitis remains.

[0016] A growing body of evidence indicates that NGF functions as a mediator of certain pain states. It has been shown that anti-NGF antibodies can produce a sustained thermal and

chemical analgesic effect, as well as block the hyperalgesia which develops from carrageenan-induced inflammation (McMahon *et al.,* Nat. Med. 1:774, 1995). Studies of a small molecule NGF receptor antagonist for blockading the bioactivity of NGF have indicated an analgesic effect on neuropathic and inflammatory pain states (Owolabi *et al.*, J. Pharmacol. Exp. Ther. 289:1271, 1999)). In the Owolabi *et al.* study, the analgesic effect of the small molecule NGF activity inhibitor may be less than that of morphine depending on the route of administration. Since opioids, such as morphine, have many unwanted side-effects, a need remains for providing analgesia in the variety of pain states mediated by NGF which allows flexibility in effective administration.

SUMMARY

[0017] The present disclosure provides various aptamers that bind to the beta subunit of nerve growth factor, referred to individually herein as a "β-NGF aptamer", and methods for using such β-NGF aptamers to treat β-NGF mediated diseases and disorders, including the treatment of pain and pruritus and pruritic conditions. Included are pharmaceutical compositions or formulations comprised of a β-NGF aptamer or a pharmaceutically acceptable salt thereof and at least one pharmaceutically acceptable carrier.

[0018] The compositions of the present disclosure can be prepared in any suitable pharmaceutically acceptable dosage form. The formulations and dosages described herein are designed to maximize clinical efficacy in the treatment of various conditions, such as pain and pruritus and pruritic conditions, while simultaneously decreasing or minimizing adverse side effects.

[0019] The present disclosure further provides methods for preventing, treating or ameliorating a disease or condition mediated by β-NGF, the methods comprising administering a β-NGF aptamer or a pharmaceutical composition of the β-NGF aptamer to a vertebrate, specifically a mammal, more specifically a human. Specifically, the present disclosure provides methods for treating, preventing or ameliorating pain and pruritus and pruritic conditions. In some aspects, the β-NGF mediated disease or condition is one in which β-NGF activity may directly or indirectly lead to pruritus at some stage of the disease. In some embodiments the disease or condition to be treated, prevented or ameliorated is dermatitis or eczema. In other

embodiments, the disease or condition to be treated, prevented or ameliorated is atopic dermatitis.

[0020] In one embodiment, a therapeutic effect (*e.g.*, treating, preventing or ameliorating pain and pruritus and pruritic conditions) may be achieved by administering a β-NGF aptamer such that the aptamer is exposed to, and can bind to, β-NGF regardless of the method of delivery of the aptamer to the patient being treated. In a related embodiment, the therapeutic effect may be achieved by the administration of the β-NGF aptamer such that it is exposed to, and binds to, β-NGF and thereby prevents or reduces the binding of β-NGF to one or more of its various cell receptors. In one embodiment, the cell receptor is p75. In another embodiment, the cell receptor is a Trk receptor. In yet another embodiment, the cell receptor is TrkA. In yet another embodiment, the β-NGF aptamer prevents or reduces the level of phosphorylation of the β-NGF receptor and other internal cellular proteins.

[0021] The provided methods encompass administration of the β-NGF aptamer in association with one or more secondary active agents. Such administration can be sequential or in a combination composition.

[0022] In another aspect, the present disclosure provides an *in vitro* diagnostic method comprising contacting a β-NGF aptamer with a sample suspected of comprising β-NGF. In another aspect, the present disclosure provides an *in vivo* diagnostic method comprising providing a suitably labeled β-NGF aptamer, injecting the aptamer into an individual suspected of having β-NGF-mediated disease or disorder, and detecting the labeled aptamer for the purpose of diagnosing or evaluating the health status of the individual. The label used will be selected in accordance with the imaging modality to be used.

BRIEF DESCRIPTION OF THE DRAWINGS

[0023] Figure 1 illustrates the binding curves for aptamer 2426-66 (■) (SEQ ID NO: 1) compared to the random library (○).

[0024] Figures 2A and 2B depict aptamer consensus sequences identified using 454 sequencing for aptamer 2426-66 (SEQ ID NO: 1).

[0025] Figure 3 illustrates dimerization strategy # 1 for a β-NGF aptamer.

[0026] Figure 4 illustrates dimerization strategy #2 for a β-NGF aptamer.

[0027] Figure 5 illustrates graphically the ability of various aptamers to inhibit human β-NGF induced differentiation of PC12 cells as tested in the neurite growth assay described in Example 4.

[0028] Figure 6 illustrates graphically the inhibition of β-NGF mediated neurite growth as a function of aptamer concentration for aptamer 2426-66 (■) (SEQ ID NO: 1), and its truncated variant 2426-66-50 (○) (SEQ ID NO: 2), measured as described in Example 4.

[0029] Figure 7 illustrates graphically the inhibition of human β-NGF, mouse β- NGF and rat β-NGF mediated neurite growth by aptamer 2426-66 (SEQ ID NO: 1) and truncated variants 2426-66-50 (SEQ ID NO: 2) and 2426-66-53 (SEQ ID NO: 43). All three aptamers inhibited mouse β-NGF nearly as effectively as human β-NGF, and inhibited rat β-NGF to a lesser extent.

[0030] Figure 8 illustrates graphically the results of a TrkA phosphorylation assay using aptamers 2426-66 (SEQ ID NO: 1) and truncated variants 2426-66-50 (SEQ ID NO: 2) and 2426-66-3 (SEQ ID NO: 5).

[0031] Figure 9 illustrates graphically a TrkA phosphorylation assay for truncated aptamer 2426-66-50 (SEQ ID NO: 2) using mouse and rate β-NGF.

[0032] Figure 10 depicts C-5 pyrimidine modifications used to prepare the aptamers described herein.

[0033] Figure 11 illustrates graphically the reduction of scratching frequency over four weeks in diseased mice treated with aptamer 2426-66-50 (SEQ ID NO: 2) (●), but not in untreated mice (▪) or mice treated with hydrophilic ointment (HO) (▲), as described in Example 5. Statistically significant differences ($p < 0.05$) were observed between aptamer treatment and no treatment (*), or aptamer treatment and HO treatment (#), as determined by t-test.

[0034] Figure 12 illustrates graphically the reduction of clinical skin condition score over four weeks in diseased mice treated with aptamer 2426-66-50 (SEQ ID NO: 2), (●), but not in untreated mice (▪) or mice treated with HO (▲), as described in Example 5. Statistically significant differences ($p < 0.05$) were observed between aptamer treatment and no treatment (*), or aptamer treatment and HO treatment (#), as determined by t-test.

DETAILED DESCRIPTION

[0035] Reference will now be made in detail to representative embodiments of the invention. While the invention will be described in conjunction with the enumerated embodiments, it will be understood that the invention is not intended to be limited to those embodiments. On the contrary, the invention is intended to cover all alternatives, modifications, and equivalents that may be included within the scope of the present invention as defined by the claims.

[0036] One skilled in the art will recognize many methods and materials similar or equivalent to those described herein, which could be used in and are within the scope of the practice of the present invention. The present invention is in no way limited to the methods and materials described.

[0037] Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art(s) to which this invention belongs. Although any methods, devices, and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods, devices and materials are now described.

[0038] All publications, published patent documents, and patent applications cited in this disclosure are indicative of the level of skill in the art(s) to which the disclosure pertains. All publications, published patent documents, and patent applications cited herein are hereby incorporated by reference to the same extent as though each individual publication, published patent document, or patent application was specifically and individually indicated as being incorporated by reference.

[0039] As used in this disclosure, including the appended claims, the singular forms "a," "an," and "the" include plural references, unless the content clearly dictates otherwise, and are used interchangeably with "at least one" and "one or more." Thus, reference to "an aptamer" includes mixtures of aptamers, and the like.

[0040] As used herein, the term "about" represents an insignificant modification or variation of the numerical value such that the basic function of the item to which the numerical value relates is unchanged.

[0041] The term "each" when used herein to refer to a plurality of items is intended to refer to at least two of the items. It need not require that all of the items forming the plurality satisfy an associated additional limitation.

[0042] As used herein, the terms "comprises," "comprising," "includes," "including," "contains," "containing," and any variations thereof, are intended to cover a non-exclusive inclusion, such that a process, method, product-by-process, or composition of matter that comprises, includes, or contains an element or list of elements does not include only those elements but may include other elements not expressly listed or inherent to such process, method, product-by-process, or composition of matter.

[0043] As used herein, the term "nucleotide" refers to a ribonucleotide or a deoxyribonucleotide, or a modified form thereof, as well as an analog thereof. Nucleotides include species that include purines (*e.g.*, adenine, hypoxanthine, guanine, and their derivatives and analogs) as well as pyrimidines (*e.g.*, cytosine, uracil, thymine, and their derivatives and analogs).

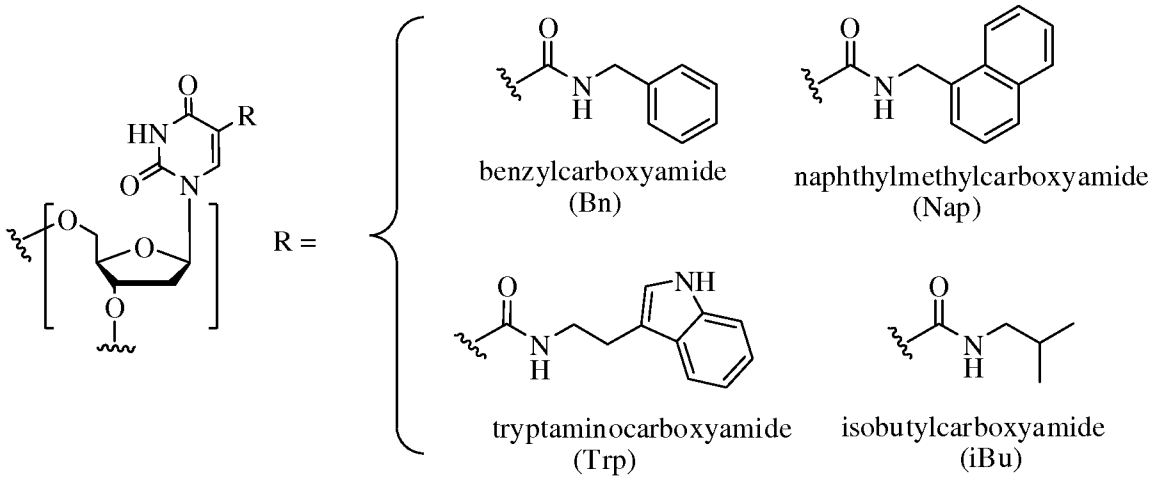
[0044] As used herein, "nucleic acid," "oligonucleotide," and "polynucleotide" are used interchangeably to refer to a polymer of nucleotides and include DNA, RNA, DNA/RNA hybrids and modifications of these kinds of nucleic acids, oligonucleotides and polynucleotides, wherein the attachment of various entities or moieties to the nucleotide units at any position are included. The terms "polynucleotide," "oligonucleotide," and "nucleic acid" include double- or single-stranded molecules as well as triple-helical molecules. Nucleic acid, oligonucleotide, and polynucleotide are broader terms than the term aptamer and, thus, the terms nucleic acid, oligonucleotide, and polynucleotide include polymers of nucleotides that are aptamers but the terms nucleic acid, oligonucleotide, and polynucleotide are not limited to aptamers.

[0045] As used herein, the terms "modify", "modified", "modification", and any variations thereof, when used in reference to an oligonucleotide, means that at least one of the four constituent nucleotide bases (*i.e.*, A, G, T/U, and C) of the oligonucleotide is an analog or ester of a naturally occurring nucleotide. In some embodiments, the modified nucleotide confers nuclease resistance to the oligonucleotide. A pyrimidine with a substitution at the C-5 position is an example of a modified nucleotide. Modifications can include backbone modifications, methylations, unusual base-pairing combinations such as the isobases isocytidine and

isoguanidine, and the like. Modifications can also include 3' and 5' modifications, such as capping. Other modifications can include substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (*e.g.*, methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, *etc.*) and those with charged linkages (*e.g.*, phosphorothioates, phosphorodithioates, *etc.*), those with intercalators (*e.g.*, acridine, psoralen, *etc.*), those containing chelators (*e.g.*, metals, radioactive metals, boron, oxidative metals, *etc.*), those containing alkylators, and those with modified linkages (*e.g.*, alpha anomeric nucleic acids, *etc.*). Further, any of the hydroxyl groups ordinarily present on the sugar of a nucleotide may be replaced by a phosphonate group or a phosphate group; protected by standard protecting groups; or activated to prepare additional linkages to additional nucleotides or to a solid support. The 5′ and 3′ terminal OH groups can be phosphorylated or substituted with amines, organic capping group moieties of from about 1 to about 20 carbon atoms, polyethylene glycol (PEG) polymers in one embodiment ranging from about 10 to about 80 kDa, PEG polymers in another embodiment ranging from about 20 to about 60 kDa, or other hydrophilic or hydrophobic biological or synthetic polymers. In one embodiment, modifications are of the C-5 position of pyrimidines. These modifications can be produced through an amide linkage directly at the C-5 position or by other types of linkages.

[0046] Polynucleotides can also contain analogous forms of ribose or deoxyribose sugars that are generally known in the art, including 2'-O-methyl-, 2'-O-allyl, 2'-fluoro- or 2'-azido-ribose, carbocyclic sugar analogs, α-anomeric sugars, epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, sedoheptuloses, acyclic analogs and abasic nucleoside analogs such as methyl riboside. As noted above, one or more phosphodiester linkages may be replaced by alternative linking groups. These alternative linking groups include embodiments wherein phosphate is replaced by P(O)S ("thioate"), P(S)S ("dithioate"), (O)NR_2 ("amidate"), P(O)R, P(O)OR', CO or CH_2 ("formacetal"), in which each R or R' is independently H or substituted or unsubstituted alkyl (1-20 C) optionally containing an ether (-O-) linkage, aryl, alkenyl, cycloalky, cycloalkenyl or araldyl. Not all linkages in a polynucleotide need be identical. Substitution of analogous forms of sugars, purines, and pyrimidines can be advantageous in designing a final product, as can alternative backbone structures like a polyamide backbone, for example.

[0047] As used herein, the term "C-5 modified pyrimidine" refers to a pyrimidine with a modification at the C-5 position including, but not limited to, those moieties illustrated in Figure 10. Examples of a C-5 modified pyrimidine include those described in U.S. Pat. Nos. 5,719,273 and 5,945,527, as well as, U.S. Provisional Application Serial No. 61/264,545, filed November 25, 2009, entitled "Nuclease Resistant Oligonucleotides." Examples of a C-5 modification include substitution of deoxyuridine at the C-5 position with a substituent independently selected from: benzylcarboxyamide (alternatively benzylaminocarbonyl) (Bn), naphthylmethylcarboxyamide (alternatively naphthylmethylaminocarbonyl) (Nap), tryptaminocarboxyamide (alternatively tryptaminocarbonyl) (Trp), and isobutylcarboxyamide (alternatively isobutylaminocarbonyl) (iBu) as illustrated immediately below.



[0048] Chemical modifications of a C-5 modified pyrimidine can also be combined with, singly or in any combination, 2'-position sugar modifications, modifications at exocyclic amines, and substitution of 4-thiouridine and the like.

[0049] Representative C-5 modified pyrimidines include: 5-(N-benzylcarboxyamide)-2'-deoxyuridine (BndU), 5-(N-benzylcarboxyamide)-2'-O-methyluridine, 5-(N-benzylcarboxyamide)-2'-fluorouridine, 5-(N-isobutylcarboxyamide)-2'-deoxyuridine (iBudU), 5-(N-isobutylcarboxyamide)-2'-O-methyluridine, 5-(N-isobutylcarboxyamide)-2'-fluorouridine, 5-(N-tryptaminocarboxyamide)-2'-deoxyuridine (TrpdU), 5-(N-tryptaminocarboxyamide)-2'-O-methyluridine, 5-(N-tryptaminocarboxyamide)-2'-fluorouridine, 5-(N-[1-(3-trimethylamonium) propyl]carboxyamide)-2'-deoxyuridine chloride, 5-(N-naphthylmethylcarboxyamide)-2'-

deoxyuridine (NapdU), 5-(N-naphthylmethylcarboxyamide)-2'-O-methyluridine, 5-(N-naphthylmethylcarboxyamide)-2'-fluorouridine or 5-(N-[1-(2,3-dihydroxypropyl)]carboxyamide)-2'-deoxyuridine).

[0050] If present, a modification to the nucleotide structure can be imparted before or after assembly of a polymer. A sequence of nucleotides can be interrupted by non-nucleotide components. A polynucleotide can be further modified after polymerization, such as by conjugation with a labeling component.

[0051] As used herein, the term "at least one pyrimidine," when referring to modifications of a nucleic acid, refers to one, several, or all pyrimidines in the nucleic acid, indicating that any or all occurrences of any or all of C, T, or U in a nucleic acid may be modified or not.

[0052] As used herein, "nucleic acid ligand," "aptamer," and "clone" are used interchangeably to refer to a non-naturally occurring nucleic acid that has a desirable action on a target molecule. A desirable action includes, but is not limited to, binding of the target, catalytically changing the target, reacting with the target in a way that modifies or alters the target or the functional activity of the target, covalently attaching to the target (as in a suicide inhibitor), and facilitating the reaction between the target and another molecule. In one embodiment, the action is specific binding affinity for a target molecule, such target molecule being a three dimensional chemical structure other than a polynucleotide that binds to the nucleic acid ligand through a mechanism which is independent of Watson/Crick base pairing or triple helix formation, wherein the aptamer is not a nucleic acid having the known physiological function of being bound by the target molecule. Aptamers to a given target include nucleic acids that are identified from a candidate mixture of nucleic acids, where the aptamer is a ligand of the target, by a method comprising: (a) contacting the candidate mixture with the target, wherein nucleic acids having an increased affinity to the target relative to other nucleic acids in the candidate mixture can be partitioned from the remainder of the candidate mixture; (b) partitioning the increased affinity nucleic acids from the remainder of the candidate mixture; and (c) amplifying the increased affinity nucleic acids to yield a ligand-enriched mixture of nucleic acids, whereby aptamers of the target molecule are identified. It is recognized that affinity interactions are a matter of degree; however, in this context, the "specific binding affinity" of an

aptamer for its target means that the aptamer binds to its target generally with a much higher degree of affinity than it binds to other, non-target, components in a mixture or sample. An "aptamer" or "nucleic acid ligand" is a set of copies of one type or species of nucleic acid molecule that has a particular nucleotide sequence. An aptamer can include any suitable number of nucleotides. "Aptamers" refer to more than one such set of molecules. Different aptamers can have either the same or different numbers of nucleotides. Aptamers may be DNA or RNA and may be single stranded, double stranded, or contain double stranded or triple stranded regions.

[0053] As used herein, "protein" is used synonymously with "peptide," "polypeptide," or "peptide fragment." A "purified" polypeptide, protein, peptide, or peptide fragment is substantially free of cellular material or other contaminating proteins from the cell, tissue, or cell-free source from which the amino acid sequence is obtained, or substantially free from chemical precursors or other chemicals when chemically synthesized.

[0054] As used herein, "modulate" means to alter, either by increasing or decreasing, the level of a peptide or polypeptide, or to alter, either by increasing or decreasing, the stability or activity of a peptide or a polypeptide. The term "inhibit" means to decrease the level of a peptide or a polypeptide or to decrease the stability or activity of a peptide or a polypeptide. As described herein, the protein which is modulated or inhibited is β-NGF.

[0055] As used herein, the term "bioactivity" indicates an effect on one or more cellular or extracellular process (*e.g.*, via binding, signaling, etc.) which can impact physiological or pathophysiological processes.

[0056] As used herein, the terms "nerve growth factor," "NGF," and "β-NGF" refer to the beta subunit of nerve growth factor and variants thereof that retain at least part of the activity of NGF. As used herein, NGF includes all mammalian species of native sequence NGF, including human, canine, feline, murine, primate, equine, and bovine.

[0057] As used herein, "NGF receptor" refers to a polypeptide that is bound by or activated by NGF. NGF receptors include the TrkA receptor and the p75 receptor of any mammalian species, including, but are not limited to, human, canine, feline, murine, equine, primate, and bovine.

[0058] A "β-NGF aptamer" is an aptamer that is capable of binding to and modifying the activity of β-NGF. As used herein, a "β-NGF aptamer" refers to an aptamer which is able to

bind to β-NGF and/or inhibit β-NGF biological activity and/or downstream pathway(s) mediated by NGF signaling.

[0059] As used herein, "disease or medical condition mediated by β-NGF" refers to diseases or medical conditions in which β-NGF activity may directly or indirectly lead to pain or pruritus at some stage in the disease process, including any of the diseases or medical conditions listed in Table 7. Thus, treatment with a β-NGF aptamer inhibits the pain or pruritus that occurs due to β-NGF activity in these diseases or medical conditions. The aptamer to β-NGF may further block the binding of β-NGF to one or more of its receptors.

[0060] As used herein, "pain" refers to acute pain, chronic pain, nociceptive pain, visceral pain, somatic pain, non-nociceptive pain, neuropathic pain, sympathetic pain, or to pain related to β-NGF mediated inflammation processes.

[0061] The term "pruritus" refers to itching which can range from a mild sensation to an intense sensation of itching pain. The itching may accompany primary skin disease or may be a symptom of systemic disease--sometimes the only symptom. Skin diseases in which itching can be most severe include, among others, scabies, pediculosis, insect bites, xerosis, urticaria, atopic dermatitis, contact dermatitis, lichen planus, miliaria and dermatitis herpetiformis. Systemic causes of pruritus include chronic hepatic or renal disease and lymphoma.

[0062] The terms "skin disorder" and "skin disease" refer to any disease or condition that affects or involves the skin, including skin conditions such as atopic dermatitis, ichthyosis, xeroderma, seborrheic dermatitis, allergic contact dermatitis, alopecia, pemphigus, dermatitis herpetiformis, psoriasis, candidiasis, acne, dermatophytosis, diaper rash, cradle cap, eczema, hookworm and skin damage from, *e.g.*, wounds, burns, and fecal and urinary incontinence.

[0063] As utilized herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of a federal or a state government or listed in the U.S. Pharmacopoeia or other generally recognized pharmacopoeia for use in animals and, more particularly, in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered and includes, but is not limited to, such sterile liquids as water and oils.

[0064] A "pharmaceutically acceptable salt" or "salt" of a β-NGF aptamer is a product of the disclosed compound that contains an ionic bond and is typically produced by reacting the

disclosed compound with either an acid or a base, suitable for administering to an individual. A pharmaceutically acceptable salt can include, but is not limited to, acid addition salts including hydrochlorides, hydrobromides, phosphates, sulphates, hydrogen sulphates, alkylsulphonates, arylsulphonates, arylalkylsulfonates, acetates, benzoates, citrates, maleates, fumarates, succinates, lactates, and tartrates; alkali metal cations such as Li, Na, K, alkali earth metal salts such as Mg or Ca, or organic amine salts.

[0065] A "pharmaceutical composition" is a formulation comprising a β-NGF aptamer in a form suitable for administration to an individual. A pharmaceutical composition is typically formulated to be compatible with its intended route of administration. Examples of routes of administration include, but are not limited to, oral and parenteral, *e.g.*, intravenous, intradermal, subcutaneous, inhalation, topical, transdermal, transmucosal, and rectal administration.

[0066] As used herein, the term "therapeutically effective amount" generally means the amount necessary to ameliorate at least one symptom of a disorder or condition to be prevented, reduced, or treated as described herein. The phrase "therapeutically effective amount" as it relates to the β-NGF aptamers of the present disclosure means the aptamer dosage that provides the specific pharmacological response for which the aptamer is administered in a significant number of individuals in need of such treatment. It is emphasized that a therapeutically effective amount of an aptamer that is administered to a particular individual in a particular instance will not always be effective in treating the conditions/diseases described herein, even though such dosage is deemed to be a therapeutically effective amount by those of skill in the art.

The SELEX Method

[0067] The terms "SELEX" and "SELEX process" are used interchangeably herein to refer generally to a combination of (1) the selection of nucleic acids that interact with a target molecule in a desirable manner, for example binding with high affinity to a protein, with (2) the amplification of those selected nucleic acids. The SELEX process can be used to identify aptamers with high affinity to a specific target molecule or biomarker.

[0068] SELEX generally includes preparing a candidate mixture of nucleic acids, binding of the candidate mixture to the desired target molecule to form an affinity complex, separating the affinity complexes from the unbound candidate nucleic acids, separating and isolating the

nucleic acid from the affinity complex, purifying the nucleic acid, and identifying a specific aptamer sequence. The process may include multiple rounds to further refine the affinity of the selected aptamer. The process can include amplification steps at one or more points in the process. See, *e.g.,* U.S. Patent No. 5,475,096, entitled "Nucleic Acid Ligands." The SELEX process can be used to generate an aptamer that covalently binds its target as well as an aptamer that non-covalently binds its target. See, *e.g.*, U.S. Patent No. 5,705,337 entitled "Systematic Evolution of Nucleic Acid Ligands by Exponential Enrichment: Chemi-SELEX."

[0069] The SELEX process can be used to identify high-affinity aptamers containing modified nucleotides that confer improved characteristics on the aptamer, such as, for example, improved *in vivo* stability or improved delivery characteristics. Examples of such modifications include chemical substitutions at the ribose and/or phosphate and/or base positions. SELEX process-identified aptamers containing modified nucleotides are described in U.S. Patent No. 5,660,985, entitled "High Affinity Nucleic Acid Ligands Containing Modified Nucleotides," which describes oligonucleotides containing nucleotide derivatives chemically modified at the 5'- and 2'-positions of pyrimidines. U.S. Patent No. 5,580,737, see supra, describes highly specific aptamers containing one or more nucleotides modified with 2'-amino (2'-NH_2), 2'-fluoro (2'-F), and/or 2'-O-methyl (2'-OMe). See also, U.S. Patent Application Publication No. 20090098549, entitled "SELEX and PHOTOSELEX," which describes nucleic acid libraries having expanded physical and chemical properties and their use in SELEX and photoSELEX.

[0070] U.S. Provisional Application Serial No. 61/264,545, filed November 25, 2009, entitled "Nuclease Resistant Oligonucleotides," describes methods for producing oligonucleotides with improved nuclease resistance. The nuclease resistant oligonucleotides include at least one pyrimidine modified at the C-5 position with a group selected from those set forth in Figure 10. In various embodiments, the modifications include substitution of deoxyuridine at the C-5 position with a substituent independently selected from: benzylcarboxyamide (Bn), naphthylmethylcarboxyamide (Nap), tryptaminocarboxyamide (Trp), and isobutylcarboxyamide as illustrated above.

[0071] SELEX can also be used to identify aptamers that have desirable off-rate characteristics. See U.S. Patent Publication No. 20090004667, entitled "Method for Generating Aptamers with Improved Off-Rates," which describes improved SELEX methods for generating

aptamers that can bind to target molecules. Methods for producing aptamers and photoaptamers having slower rates of dissociation from their respective target molecules are described. The methods involve contacting the candidate mixture with the target molecule, allowing the formation of nucleic acid-target complexes to occur, and performing a slow off-rate enrichment process wherein nucleic acid-target complexes with fast dissociation rates dissociate and do not reform, while complexes with slow dissociation rates remain intact. Additionally, the methods include the use of modified nucleotides in the production of candidate nucleic acid mixtures to generate aptamers with improved off-rate performance (see U.S. Patent Publication No. 20090098549, entitled "SELEX and PhotoSELEX").

[0072] "Target" or "target molecule" or "target" refers herein to any compound upon which a nucleic acid can act in a desirable manner. A target molecule can be a protein, peptide, nucleic acid, carbohydrate, lipid, polysaccharide, glycoprotein, hormone, receptor, antigen, antibody, virus, pathogen, toxic substance, substrate, metabolite, transition state analog, cofactor, inhibitor, drug, dye, nutrient, growth factor, cell, tissue, any portion or fragment of any of the foregoing, *etc.*, without limitation. Virtually any chemical or biological effector may be a suitable target. Molecules of any size can serve as targets. A target can also be modified in certain ways to enhance the likelihood or strength of an interaction between the target and the nucleic acid. A target can also include any minor variation of a particular compound or molecule, such as, in the case of a protein, for example, minor variations in amino acid sequence, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labeling component, which does not substantially alter the identity of the molecule. A "target molecule" or "target" is a set of copies of one type or species of molecule or multimolecular structure that is capable of binding to an aptamer. "Target molecules" or "targets" refer to more than one such set of molecules. Embodiments of the SELEX process in which the target is a peptide are described in U.S. Patent No. 6,376,190, entitled "Modified SELEX Processes Without Purified Protein." In the instant case, the target is β-NGF.

Aptamers

[0073] The aptamers of the instant disclosure were identified using the improved SELEX method for identifying aptamers having slow off-rates as described in Example 1, which describes a representative method for the selection and production of a DNA aptamer to β-NGF. The form of β-NGF used in the selection process was a recombinant human protein and was isolated as the monomeric form of the protein with a molecular weight of 13.2 kD. In solution the monomer forms a dimer. Using this method, the DNA aptamer to β-NGF designated as aptamer 2426-66 (SEQ ID NO: 1) was identified.

[0074] Using aptamer 2426-66 (SEQ ID NO: 1), studies were conducted to identify the minimum sequence length required to maintain strong affinity for β-NGF as described in Example 2. Minimizing the sequence length allows for more reproducible aptamer synthesis in a chemical process and potentially aids in adsorption through the skin as well as incorporation into a pharmaceutical formulation. The truncation studies led to the identification of aptamers having a number of truncated sequences that were also avid binders to β-NGF, with K_d values up to about 30 nM. These sequences include SEQ ID NOS: 1, 2, 9-44, and 149 (Tables 3 and 4). In particular aptamer 2426-66-50 (SEQ ID NO: 2; Table 4), a 28-mer having a K_d of 1.4 nM for β-NGF was identified.

[0075] Additional sequencing studies were conducted on the sequence pool from which 2426-66 (SEQ ID NO: 1) was selected. The sequencing method used was 454 Sequencing. This is a large-scale, high throughput method that uses parallel pyrosequencing. The method provides unbiased sample preparation and very accurate sequence analysis. In this method, biotinylated DNA fragments are captured on streptavidin beads and then amplified by PCR. The unbiotinylated strand is released from the bead and used as a single stranded template DNA library. This library is then amplified by PCR. Each bead then contains amplified, clonal copies of the DNA fragments. This library of beads is then used in an enzymatic sequencing process. The sequencing data was used to identify a consensus sequence for a β-NGF aptamer. Furthermore, nucleotide substitution studies described in Example 3 led to the discovery that seven of nine BndU positions in the consensus sequence were desirable for β-NGF binding, but two BndU positions could be replaced with dT with no loss of binding activity. The consensus

sequence is shown in Figure 2A, along with a graphic representation of the nucleotide frequency at each position relative to the 2426-66 (SEQ ID NO: 1) aptamer. As illustrated in Figure 2A, the consensus sequence is:

BAZGRGGRSZWGGGGZZWADCCGZZRZG (SEQ ID NO: 45)

wherein

B is selected from any nucleotide other than A;

R is independently selected from an A or G;

S is selected from a C or G;

W is independently selected from a Z or T;

D is selected from any nucleotide other than C; and

Z is independently selected from a modified nucleotide, specifically a modified pyrimidine.

[0076] In one aspect, B is selected from a C, G or Z; D is selected from an A, G or Z and R, S, W and Z are as defined above.

[0077] In another aspect, the consensus sequence is:

BAZGRGGRSZZGGGGZZZADCCGZZRZG (SEQ ID NO: 3)

wherein B, R, S, D and Z are as defined above.

[0078] In some embodiments, Z is a modified uridine. In other embodiments, Z is C-5 modified pyrimidine as defined above. In yet other embodiments, Z is a C-5 modified pyrimidine independently selected from the group consisting of 5-(N-benzylcarboxyamide)-2'-deoxyuridine (BndU), 5-(N-benzylcarboxyamide)-2'-O-methyluridine, 5-(N-benzylcarboxyamide)-2'-fluorouridine, 5-(N-isobutylcarboxyamide)-2'-deoxyuridine (iBudU), 5-(N-isobutylcarboxyamide)-2'-O-methyluridine, 5-(N-isobutylcarboxyamide)-2'-fluorouridine, 5-(N-tryptaminocarboxyamide)-2'-deoxyuridine (TrpdU), 5-(N-tryptaminocarboxyamide)-2'-O-methyluridine, 5-(N-tryptaminocarboxyamide)-2'-fluorouridine, 5-(N-[1-(3-trimethylamonium) propyl]carboxyamide)-2'-deoxyuridine chloride, 5-(N-naphthylmethylcarboxyamide)-2'-deoxyuridine (NapdU), 5-(N-naphthylmethylcarboxyamide)-2'-O-methyluridine, 5-(N-naphthylmethylcarboxyamide)-2'-fluorouridine and 5-(N-[1-(2,3-dihydroxypropyl)]carboxyamide)-2'-deoxyuridine). In other embodiments, Z is 5-(N-benzylcarboxyamide)-2'-deoxyuridine (BndU). Any of these nucleotide modifications are

anticipated to be equally effective in promoting high affinity binding to β-NGF and providing a slow off rate.

[0079] As used herein, "consensus sequence", when used in reference to a series of related nucleic acids, refers to a nucleotide sequence that reflects the most common choice of base at each position in the sequence where the series of related nucleic acids has been subjected to intensive mathematical and/or sequence analysis.

[0080] The present disclosure provides β-NGF aptamers identified using the SELEX method and listed in Tables 3 and 4 (SEQ ID NOS: 1, 2, 9-44 and 149). Aptamers to β-NGF that are substantially homologous to any of the listed aptamers and that have a substantially similar ability to bind β-NGF as that of an aptamer selected from the group of aptamers set forth in Tables 3 and 4 (SEQ ID NOS: 1, 2, 9-44 and 149) are also encompassed by the present disclosure. Further, aptamers to β-NGF that have substantially the same structural form as the aptamers identified herein and that have a substantially similar ability to bind β-NGF as that of an aptamer selected from the group of aptamers set forth in Tables 3 and 4 (SEQ ID NOS: 1, 2, 9-44 and 149) are also encompassed by the present disclosure.

[0081] In one aspect, the present disclosure provides an aptamer that specifically binds to β-NGF and includes a primary nucleic acid sequence. In one embodiment, the primary nucleic acid sequence is selected from SEQ ID NOS: 1, 2, 9-44 and 149. In other embodiments, the primary nucleic acid sequence is selected such that it is at least about 75% identical, at least about 80% identical, at least about 85% identical, at least about 90% identical, or at least about 95% identical to a primary nucleic acid sequence selected from SEQ ID NOS: 1, 2, 9-44 and 149.

[0082] The terms "sequence identity", "percent sequence identity", "percent identity", "% identical", "% identity", and variations thereof, when used in the context of two or more nucleic acid sequences, are used interchangeably to refer to two or more sequences or subsequences that are the same or have a specified percentage of nucleotides that are the same, when compared and aligned for maximum correspondence, as measured using a sequence comparison algorithm or by visual inspection. For sequence comparisons, typically one sequence acts as a reference sequence to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated if

necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters. Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith and Waterman, Adv. Appl. Math., 2:482, 1981, by the homology alignment algorithm of Needleman and Wunsch, J. Mol. Biol., 48:443, 1970, by the search for similarity method of Pearson and Lipman, Proc. Nat'l. Acad. Sci. USA 85:2444, 1988, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by visual inspection (see generally, Ausubel, F. M. *et al.*, Current Protocols in Molecular Biology, pub. by Greene Publishing Assoc. and Wiley-Interscience (1987)).

[0083] One example of an algorithm that is suitable for determining percent sequence identity is the algorithm used in the basic local alignment search tool (hereinafter "BLAST"), see, e.g. Altschul *et al.*, J. Mol. Biol. 215:403-410, 1990 and Altschul *et al.*, Nucleic Acids Res., 15:3389-3402, 1997. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (hereinafter "NCBI"). The default parameters used in determining sequence identity using the software available from NCBI, *e.g.*, BLASTN (for nucleotide sequences) are described in McGinnis *et al.*, Nucleic Acids Res., 32:W20-W25, 2004.

[0084] As used herein, when describing the percent identity of a nucleic acid, such as a β-NGF aptamer, the sequence of which is at least, for example, about 95% identical to a reference nucleotide sequence, it is intended that the nucleic acid sequence is identical to the reference sequence except that the nucleic acid sequence may include up to five point mutations per each 100 nucleotides of the reference nucleic acid sequence. In other words, to obtain a desired nucleic acid sequence, the sequence of which is at least about 95% identical to a reference nucleic acid sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or some number of nucleotides up to 5% of the total number of nucleotides in the reference sequence may be inserted into the reference sequence (referred to herein as an insertion). These mutations of the reference sequence to generate the desired sequence may occur at the 5′ or 3′ terminal positions of the reference

nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence. The reference (query) sequence may be any one of the entire nucleotide sequences shown in SEQ ID NOS: 1, 2, 9-44 and 149, or any fragment of any of these sequences.

[0085] In one aspect, each of the consensus sequences of SEQ ID NO: 45 or SEQ ID NO: 3 can be modified to include at least one insertion. In one embodiment, the consensus sequences of either SEQ ID NO: 45 or SEQ ID NO: 3 is modified such that one nucleotide (N) is inserted into the consensus sequence between bases 9 and 10. In another embodiment, the consensus sequence of either SEQ ID NO: 45 or SEQ ID NO: 3 is modified such that one nucleotide (N) is inserted into the consensus sequence between bases 15 and 16. In another embodiment, the consensus sequences of either SEQ ID NO: 45 or SEQ ID NO: 3 is modified such that one nucleotide (N) is inserted into the consensus sequence between bases 9 and 10 and an additional nucleotide (N) is inserted into the consensus sequence between bases 15 and 16. These embodiments are as illustrated as follows:

$$BAZGRGGRSN_{(0-1)}ZWGGGGN_{(0-1)}ZZWADCCGZZRZG \text{ (SEQ ID NO: 154)}$$

$$BAZGRGGRSN_{(0-1)}ZZGGGGN_{(0-1)}ZZZADCCGZZRZG \text{ (SEQ ID NO: 155)}$$

wherein B, R, S, D and Z are as defined above and N is independently selected from any naturally occurring or modified nucleotide (A, C, G, or T).

[0086] In another aspect, the present disclosure provides a β-NGF aptamer that, upon binding β-NGF, modulates a β-NGF function. In various embodiments, the aptamer modulates a β-NGF function *in vivo*. In various embodiments, the β-NGF aptamer includes a sequence of contiguous nucleotides that are identical to a sequence of contiguous nucleotides included in any of SEQ ID NOS: 1, 2, 9-44 and 149. In various embodiments, the sequence of contiguous nucleotides in the β-NGF aptamer can include any number of nucleotides that are identical to the same number of nucleotides in a sequence of contiguous nucleotides included in any of SEQ ID NOS: 1, 2, 9-44 and 149. In various embodiments, the sequence of contiguous nucleotides in the β-NGF aptamer includes a sequence of from about 4 to about 30 contiguous nucleotides that are identical to a sequence of from about 4 to about 30 contiguous nucleotides included in any of SEQ ID NOS: 1, 2, 9-44 and 149. In an exemplary embodiment, the β-NGF aptamer includes a

sequence of 30 contiguous nucleotides that are identical to a sequence of 30 contiguous nucleotides included in any of SEQ ID NOS: 1, 2, 9-44 and 149. In another exemplary embodiment, the β-NGF aptamer includes a sequence of 20 contiguous nucleotides that are identical to a sequence of 20 contiguous nucleotides included in any of SEQ ID NOS: 1, 2, 9-44 and 149. In yet another exemplary embodiment, the β-NGF aptamer includes a sequence of 8 contiguous nucleotides that are identical to a sequence of 8 contiguous nucleotides included in any of SEQ ID NOS: 1, 2, 9-44 and 149. In yet another exemplary embodiment, the β-NGF aptamer includes a sequence of 4 contiguous nucleotides that are identical to a sequence of 4 contiguous nucleotides included in any of SEQ ID NOS: 1, 2, 9-44 and 149.

[0087] In one embodiment, the β-NGF aptamer is SEQ ID NO: 1. In another embodiment, the β-NGF aptamer is SEQ ID NO: 2. In yet another embodiment, the β-NGF aptamer is derived from the consensus sequence of SEQ ID NO: 3. In other embodiments, the β-NGF aptamer is any of SEQ ID NOS: 9-44 and 149. In one embodiment, the β-NGF aptamer is at least about 95% identical, at least about 90% identical, at least about 85% identical, at least about 80% identical, or at least about 75% identical to any of SEQ ID NOS: 1, 2, 9-44 and 149. In another embodiment, the β-NGF aptamer includes a sequence from any of SEQ ID NOS: 1, 2, 9-44 and 149 and fragments of any of these.

[0088] The β-NGF aptamer can contain any number of nucleotides in addition to the β-NGF binding region. In various embodiments, the β-NGF aptamer can include up to about 100 nucleotides, up to about 95 nucleotides, up to about 90 nucleotides, up to about 85 nucleotides, up to about 80 nucleotides, up to about 75 nucleotides, up to about 70 nucleotides, up to about 65 nucleotides, up to about 60 nucleotides, up to about 55 nucleotides, up to about 50 nucleotides, up to about 45 nucleotides, up to about 40 nucleotides, up to about 35 nucleotides, up to about 30 nucleotides, up to about 25 nucleotides, and up to about 20 nucleotides.

[0089] In yet another embodiment, the β-NGF aptamer is selected from an aptamer that has similar binding characteristics and ability to treat β-NGF associated pain or pruritus and pruritic conditions as an aptamer selected from the group consisting of SEQ ID NOS: 1, 2, 9-44 and 149.

[0090] The β-NGF aptamer can be selected to have any suitable dissociation constant (K_d) for β-NGF. In an exemplary embodiment, the β-NGF aptamer has a dissociation constant (K_d) for β-NGF of about 10 nM or less. In another exemplary embodiment, the β-NGF aptamer has a dissociation constant (K_d) for β-NGF of about 15 nM or less. In yet another exemplary embodiment, the β-NGF aptamer has a dissociation constant (K_d) for β-NGF of about 20 nM or less. In yet another exemplary embodiment, the β-NGF aptamer has a dissociation constant (K_d) for β-NGF of about 25 nM or less. A suitable dissociation constant can be determined with a binding assay using a multi-point titration and fitting the equation $y = (max - min)(Protein)/(K_d + Protein) + min$ as described in Example 1, below. It is to be understood that the determination of dissociation constants is highly dependent upon the conditions under which they are measured and thus these numbers may vary significantly with respect to factors such as equilibration time, *etc*. In other embodiments, the β-NGF aptamer is an aptamer with a K_d that is less than or equal to the K_d of an aptamer selected from SEQ ID NOS: 1, 2, 9-44 and 149.

[0091] Aptamer 2426-66 (SEQ ID NO: 1) binds in a 1:1 stoichiometry with β-NGF monomer. Since the β-NGF forms a tight homodimer that is required for reaction with its target receptors, a more efficient inhibition of β-NGF activity might be achieved by using a dimeric or other multimeric form of the 2426-66 aptamer. Thus, in another embodiment, the β-NGF aptamer is a multimerization of any combination of the above sequences. Figures 3 and 4 illustrate potential approaches to the dimerization of the 2426-66 aptamer. The same strategies could be applied to any aptamer sequence with the appropriate binding characteristics for β-NGF. Similar approaches could also be used to create multimeric aptamers with as many copies of the aptamer sequence as desired. In this case, the 2426-66-50 (SEQ ID NO: 2) sequence of the truncated aptamer is used, but the full length 2426-66 sequence could also be utilized. Figure 3 illustrates a head to tail construct of two 2426-66 sequences with either, one or more, hexaethylene glycol (HEG) or abasic sugar phosphates as linkers between the two sections of the new aptamer sequence. Figure 4 depicts a dimerization of the 2426-66 sequence through the use of a branched phosphoramidite optionally including either hexaethylene glycol (HEG) or abasic sugar phosphates as linkers.

Pharmaceutical Compositions Including a β-NGF Aptamer

[0092] The present disclosure encompasses pharmaceutical compositions that include at least one aptamer to β-NGF and at least one pharmaceutically acceptable carrier. Suitable carriers are described in "Remington: The Science and Practice of Pharmacy, Twenty-first Edition," published by Lippincott Williams & Wilkins, which is incorporated herein by reference. Pharmaceutical compositions that include at least one aptamer to β-NGF and at least one pharmaceutically acceptable carrier may also include one or more active agents that is not a β-NGF inhibitor.

[0093] The aptamers described herein can be utilized in any pharmaceutically acceptable dosage form, including but not limited to injectable dosage forms, liquid dispersions, gels, aerosols, ointments, creams, lyophilized formulations, dry powders, tablets, capsules, controlled release formulations, fast melt formulations, delayed release formulations, extended release formulations, pulsatile release formulations, mixed immediate release and controlled release formulations, *etc.* Specifically, the aptamers described herein can be formulated: (a) for administration selected from any of oral, pulmonary, intravenous, intra-arterial, intrathecal, intra-articular, rectal, ophthalmic, colonic, parenteral, intracisternal, intravaginal, intraperitoneal, local, buccal, nasal, and topical administration; (b) into a dosage form selected from any of liquid dispersions, gels, aerosols, ointments, creams, tablets, sachets and capsules; (c) into a dosage form selected from any of lyophilized formulations, dry powders, fast melt formulations, controlled release formulations, delayed release formulations, extended release formulations, pulsatile release formulations, and mixed immediate release and controlled release formulations; or (d) any combination thereof.

[0094] Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can comprise one or more of the following components: (1) a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; (2) antibacterial agents such as benzyl alcohol or methyl parabens; (3) antioxidants such as ascorbic acid or sodium bisulfite; (4) chelating agents such as ethylenediaminetetraacetic acid; (5) buffers such as acetates, citrates or phosphates; and (5) agents for the adjustment of tonicity such as sodium chloride or dextrose. The pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. A parenteral

preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

[0095] Pharmaceutical compositions suitable for injectable use may include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL (BASF, Parsippany, N.J.) or phosphate buffered saline (PBS). In all cases, the composition should be sterile and should be fluid to the extent that easy syringability exists. The pharmaceutical composition should be stable under the conditions of manufacture and storage and should be preserved against the contaminating action of microorganisms such as bacteria and fungi. The term "stable", as used herein, means remaining in a state or condition that is suitable for administration to a patient.

[0096] The carrier can be a solvent or dispersion medium, including, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol or sorbitol, and inorganic salts such as sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

[0097] Sterile injectable solutions can be prepared by incorporating the active reagent (*e.g.*, a β-NGF aptamer) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating at least one β-NGF aptamer into a sterile vehicle that contains a basic dispersion medium and any other required ingredient. In the case of sterile powders for the preparation of sterile injectable solutions, exemplary methods of preparation include vacuum drying and freeze-drying, both of which will yield a powder of the

β-NGF aptamer plus any additional desired ingredient from a previously sterile-filtered solution thereof.

[0098] Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed, for example, in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the β-NGF aptamer can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition.

[0099] For administration by inhalation, the compounds are delivered in the form of an aerosol spray from a pressured container or dispenser that contains a suitable propellant, *e.g.*, a gas such as carbon dioxide, a nebulized liquid, or a dry powder from a suitable device. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active reagents are formulated into ointments, salves, gels, or creams as generally known in the art. The reagents can also be prepared in the form of suppositories (*e.g.*, with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

[00100] In one embodiment, the β-NGF aptamer is formulated for topical administration. As used herein "topical administration" refers to the delivery of a β-NGF aptamer to an animal by contacting, directly or otherwise, a formulation comprising the β-NGF aptamer to all or a portion of the skin (epidermis) of an animal. The term encompasses several routes of administration including, but not limited to, topical and transdermal. A common requirement for these modes of administration is efficient delivery to the target tissue or stratum. In one aspect, topical administration is used as a means to penetrate the epidermis and dermis and ultimately achieve systemic delivery of the β-NGF aptamer. In another aspect, topical administration is used as a means to selectively deliver the β-NGF aptamer to the epidermis or dermis of an animal, or to specific strata thereof.

[00101] For topical administration, the β-NGF aptamer may be formulated into pharmaceutically acceptable ointments, creams, lotions, eye ointments, eye drops, ear drops, impregnated dressings, and aerosols, medicated powders, medicated adhesives, foams, and may contain appropriate conventional additives or excipients, including, for example, preservatives or solvents to assist drug penetration, and emollients in ointments, gels, and creams. Such topical formulations may also contain compatible conventional carriers, for example ethanol or oleyl alcohol for lotions. Such carriers may constitute from about 1% to about 98% by weight of the formulation; more usually, such carriers will constitute up to about 80% by weight of the formulation. Specific formulations for the topical delivery of aptamers are described in U.S. Patent No. 6,841,539 and U.S. Publication No. 20050096287. The dosage delivered in a topical formulation is designed to accommodate the continuous delivery mode.

[00102] In one embodiment, a β-NGF aptamer is prepared with a carrier that will protect against rapid elimination from the body. For example, a controlled release formulation can be used, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc.

[00103] Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Pat. No. 4,522,811.

[00104] Additionally, suspensions of the β-NGF aptamer may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils, such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate, triglycerides, or liposomes. Non-lipid polycationic amino polymers may also be used for delivery. Optionally, the suspension may also include suitable stabilizers or agents to increase the solubility of the compounds and allow for the preparation of highly concentrated solutions.

[00105] In some cases, it may be especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage

unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of a β-NGF aptamer calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the β-NGF aptamers described herein are dictated by and directly dependent on the unique characteristics of the particular β-NGF aptamer and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active agent for the treatment of individuals.

[00106] Pharmaceutical compositions comprising at least one β-NGF aptamer can include one or more pharmaceutical excipients. Examples of such excipients include, but are not limited to, binding agents, filling agents, lubricating agents, suspending agents, sweeteners, flavoring agents, preservatives, buffers, wetting agents, disintegrants, effervescent agents, and other excipients. Such excipients are known in the art. Exemplary excipients include: (1) binding agents which include various celluloses and cross-linked polyvinylpyrrolidone, microcrystalline cellulose, such as Avicel® PH101 and Avicel® PH102, silicified microcrystalline cellulose (ProSolv SMCC™), gum tragacanth and gelatin; (2) filling agents such as various starches, lactose, lactose monohydrate, and lactose anhydrous; (3) disintegrating agents such as alginic acid, Primogel, corn starch, lightly crosslinked polyvinyl pyrrolidone, potato starch, maize starch, and modified starches, croscarmellose sodium, cross-povidone, sodium starch glycolate, and mixtures thereof; (4) lubricants, including agents that act on the flowability of a powder to be compressed, include magnesium stearate, colloidal silicon dioxide, such as Aerosil® 200, talc, stearic acid, calcium stearate, and silica gel; (5) glidants such as colloidal silicon dioxide; (6) preservatives, such as potassium sorbate, methylparaben, propylparaben, benzoic acid and its salts, other esters of parahydroxybenzoic acid such as butylparaben, alcohols such as ethyl or benzyl alcohol, phenolic compounds such as phenol, or quaternary compounds such as benzalkonium chloride; (7) diluents such as pharmaceutically acceptable inert fillers, such as microcrystalline cellulose, lactose, dibasic calcium phosphate, saccharides, and/or mixtures of any of the foregoing; examples of diluents include microcrystalline cellulose, such as Avicel® PH101 and Avicel® PH102; lactose such as lactose monohydrate, lactose anhydrous, and Pharmatose® DCL21; dibasic calcium phosphate such as Emcompress®; mannitol; starch; sorbitol; sucrose; and glucose; (8) sweetening agents, including any natural or artificial

sweetener, such as sucrose, saccharin sucrose, xylitol, sodium saccharin, cyclamate, aspartame, and acesulfame; (9) flavoring agents, such as peppermint, methyl salicylate, orange flavoring, Magnasweet® (trademark of MAFCO), bubble gum flavor, fruit flavors, and the like; and (10) effervescent agents, including effervescent couples such as an organic acid and a carbonate or bicarbonate. Suitable organic acids include, for example, citric, tartaric, malic, fumaric, adipic, succinic, and alginic acids and anhydrides and acid salts. Suitable carbonates and bicarbonates include, for example, sodium carbonate, sodium bicarbonate, potassium carbonate, potassium bicarbonate, magnesium carbonate, sodium glycine carbonate, L-lysine carbonate, and arginine carbonate. Alternatively, only the sodium bicarbonate component of the effervescent couple may be present.

[00107] In various embodiments, the formulations described herein are substantially pure. As used herein, "substantially pure" means the active ingredient (β-NGF aptamer) is the predominant species present (*i.e.*, on a molar basis it is more abundant than any other individual species in the composition). In one embodiment, a substantially purified fraction is a composition wherein the active ingredient comprises at least about 50 percent (on a molar basis) of all macromolecular species present. Generally, a substantially pure composition will include more than about 80% of all macromolecular species present in the composition. In various embodiments, a substantially pure composition will include at least about 85%, at least about 90%, at least about 95%, or at least about 99% of all macromolecular species present in the composition. In various embodiments, the active ingredient is purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods) wherein the composition consists essentially of a single macromolecular species.

Kits Comprising β-NGF Aptamer Compositions

[00108] The present disclosure provides kits comprising any of the β-NGF aptamers described herein. Such kits can comprise, for example, (1) at least one β-NGF aptamer; and (2) at least one pharmaceutically acceptable carrier, such as a solvent or solution. Additional kit components can optionally include, for example: (1) any of the pharmaceutically acceptable excipients identified herein, such as stabilizers, buffers, *etc.*, (2) at least one container, vial or similar apparatus for holding and/or mixing the kit components; and (3) delivery apparatus.

Methods of Treatment

[00109] The present disclosure provides methods of preventing or treating (*e.g.*, alleviating one or more symptoms of) medical conditions through the use of a β-NGF aptamer. The methods comprise administering a therapeutically effective amount of a β-NGF aptamer to a patient in need thereof. The described aptamers can also be used for prophylactic therapy. In some embodiments the β-NGF aptamer is administered topically.

[00110] The β-NGF aptamer used in methods of treatment can be: (1) a novel β-NGF aptamer prepared by the methods described herein, or a pharmaceutically acceptable salt thereof, or a prodrug thereof.

[00111] The individual or patient can be any animal (domestic, livestock or wild), including, but not limited to, cats, dogs, horses, pigs and cattle, and preferably human patients. As used herein, the terms patient, individual, and subject may be used interchangeably.

[00112] As used herein, "treating" describes the management and care of a patient for the purpose of treating a disease, condition, or disorder and includes the administration of a β-NGF aptamer to prevent the onset of the symptoms or complications of a disease, condition or disorder; to alleviate symptoms or complications of the disease, condition, or disorder; or to eliminate the presence of the disease, condition or disorder in the patient. More specifically, "treating" includes reversing, attenuating, alleviating, minimizing, suppressing or halting at least one deleterious symptom or effect of a disease (disorder) state, disease progression, disease causative agent (*e.g.*, bacteria or viruses), or other abnormal condition. Treatment is generally continued as long as symptoms and/or pathology ameliorate.

[00113] In various embodiments, the disclosed compositions (including topical formulations) and methods are used to treat dermatitis, which is often characterized as a superficial inflammation or rash of the skin characterized by redness, edema, oozing, crusting, scaling, and sometimes vesicles. Pruritis (itching) is common in dermatitis. Eczema is a term often used interchangeably with dermatitis. Examples of dermatitis or eczema include, for example, atopic dermatitis (also called infantile or flexural eczema), contact dermatitis (including allergic and irritant), xerotic eczema (also referred to as asteatotic eczema, craquele or craquelatum, winter itch, or pruritis hiemalis), exfoliative dermatitis, hand and foot dermatitis, neurodermatitis (e.g., lichen simplex chronicus), seborrheic dermatitis (cradle cap in infants,

dandruff), discoid eczema (also referred to as nummular eczema, exudative eczema, microbial eczema), dyshydrosis, venous eczema (gravitationa eczema, stasis dermatitis, varicose eczema stasis dermatitis, dermatitis herpetiformis (Duhring's Disease), autoeczematization (also referred to as id reaction, autosensitization), cercarial dermatitis (*e.g.*, swimmer's itch or duck itch), urushiol-induced contact dermatitis, which is also called toxicodendron dermatitis and rhus dermatitis (*e.g.*, poison oak, poison ivy, sumac), solar dermatitis, and housewife eczema.

[00114] In one embodiment, the disclosed compounds or pharmaceutically acceptable salts thereof, or prodrugs, can be administered in combination with other treatments that improve or eradicate itching. Compositions including the disclosed β-NGF aptamers may contain, for example, more than one aptamer, *e.g.*, an IgE, IL-6, and/or PAR2 aptamer and a β-NGF aptamer. In some examples, a β-NGF aptamer composition containing one or more aptamers is administered in combination with another useful anti-pruritic composition, such as, for example, an anti-histamine, an analgesic, an anticholinergics, a non-steroid anti-inflammation drug, a steroid, an anti-oxidant agent, a vitamin, a leukotriene modifier, an interleukin antagonist, a mast cell inhibitor, an anti-IgE antibody, a selective serotonin reuptake inhibitor, a 5-hydroxytryptamine receptor antagonist, an antibiotic, a calcineurin inhibitor, a histone deacetylase inhibitor, gabapentin or naloxone, in which active ingredients are present in free form or in the form of a pharmaceutically acceptable salt and, optionally, at least one pharmaceutically acceptable carrier, for systemic or topical use or administration simultaneously, separately, or sequentially, or the like. In general, the currently available dosage forms of the known therapeutic agents for use in such combinations will be suitable.

[00115] "Combination therapy" (or "co-therapy") includes the administration of a β-NGF aptamer composition and at least one second agent as part of a specific treatment regimen intended to provide the beneficial effect from the co-action of these therapeutic agents. The beneficial effect of the combination includes, but is not limited to, pharmacokinetic or pharmacodynamic co-action resulting from the combination of therapeutic agents. Administration of these therapeutic agents in combination typically is carried out over a defined time period (usually minutes, hours, days or weeks depending upon the combination selected).

[00116] "Combination therapy" may, but generally is not, intended to encompass the administration of two or more of these therapeutic agents as part of separate monotherapy

regimens that incidentally and arbitrarily result in the combinations of the present disclosure. "Combination therapy" is intended to embrace administration of these therapeutic agents in a sequential manner, that is, wherein each therapeutic agent is administered at a different time, as well as administration of these therapeutic agents, or at least two of the therapeutic agents, in a substantially simultaneous manner. Substantially simultaneous administration can be accomplished, for example, by administering to the subject a single dose having a fixed ratio of each therapeutic agent or in multiple, single doses for each of the therapeutic agents.

[00117] The dosage regimen utilizing the β-NGF aptamers is selected in accordance with a variety of factors, including, for example, type, species, age, weight, sex and medical condition of the patient; the severity of the condition to be treated; the route of administration; the renal and hepatic function of the patient; and the particular β-NGF aptamer or salt thereof employed. An ordinarily skilled physician or veterinarian can readily determine and prescribe the effective amount of the composition required to prevent, counter or arrest the progress of the condition.

[00118] In general, the dosage, *i.e.*, the therapeutically effective amount, ranges from about 1 μg to about 100 mg/kg body weight of the subject being treated, per day.

Efficacy

[00119] Example 4 illustrates the ability of various β-NGF aptamers and truncated variants thereof to inhibit human β-NGF mediated neurite growth (Figures 5-7) and to inhibit TrkA phosphorylation by β-NGF (Figures 8 and 9).

[00120] Example 5 illustrates the efficacy of β-NGF aptamers in reducing scratching frequency and improving the clinical skin condition by administering aptamer 2426-66-50 (SEQ ID NO: 2) to diseased mice. With reference to Figure 11, it can be seen that scratching frequency decreased steadily from day 14-28 in mice treated with aptamer 2426-66-50 (●), in contrast no change was observed in untreated mice (■) or mice treated with hydrophilic ointment (HO) (▲). Likewise, with reference to Figure 12, it can be seen that clinical skin condition improved over 4 weeks in diseased mice treated with aptamer 2426-66-50 (SEQ ID NO: 2) (●), and, as with scratching frequency, there was no improvement in untreated mice (■) or mice treated with HO (▲).

EXAMPLES

[00121] The following examples are provided for illustrative purposes only and are not intended to limit the scope of the invention as defined by the appended claims. All examples described herein were carried out using standard techniques, which are well known and routine to those of skill in the art. Routine molecular biology techniques described in the following examples can be carried out as described in standard laboratory manuals, such as Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 3rd. ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., (2001).

Example 1. Aptamer Selection and Sequences

[00122] **A. Preparation of Candidate Mixtures**

[00123] A candidate mixture of partially randomized ssDNA oligonucleotides was prepared by polymerase extension of a DNA primer annealed to a biotinylated ssDNA template as shown in Table 1. The candidate mixture contained a 40 nucleotide randomized cassette containing dATP, dGTP, dCTP and BndUTP (5-(N-benzylcarboxyamide-2'-deoxyuridine triphosphate).

[00124] 4.8 nmol of Primer 1 (SEQ ID NO:165) possessing a unique chromophore, nitroazidoaniline (ANA, designated as X in the sequence) at the 5' terminus and 4 nmol of Template 1 (SEQ ID NO: 46) possessing two biotin residues (designated as B' in the sequence) and 40 randomized positions (A, C, G, or T)(designated as N in the sequence) were combined in 100 µL 1X KOD DNA Polymerase Buffer (Novagen), heated to 95°C for 8 minutes, and cooled on ice. The 100 µL primer:template mixture was added to a 400 µL extension reaction containing 1X KOD DNA Polymerase Buffer, 0.125 U/ µL KOD XL DNA Polymerase, and 0.5 mM each dATP, dCTP, dGTP, and BndUTP , and incubated at 70°C for 30 minutes. Double-stranded product was captured via the template strand biotins by adding 1 mL streptavidin-coated magnetic beads (MagnaBind Streptavidin, Pierce, 5 mg/mL in 3 M NaCl containing 0.05% TWEEN-20) and incubating at 25°C for 10 minutes with mixing. Beads were washed three times with 0.75 mL SB17T Buffer (40 mM HEPES, pH 7.5, 125 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 0.05% TWEEN-20). The aptamer strand was eluted from the beads with 1.2 mL 20 mM NaOH, neutralized with 0.3 mL 80 mM HCl, and buffered with 15 µL 1 M HEPES, pH 7.5.

The candidate mixtures was concentrated with a Centricon-30 to approximately 0.2 mL, and quantified by UV absorbance spectroscopy.

Table 1. Sequences of SELEX Template and Primers

Oligonucleotide Designation	Sequence (5′→ 3′)	SEQ ID NO:
Template 1	AB'AB'TTTTTTTTGGTCTTGTTTCTTCTCTGTG-$(N)_{40}$-CAGGCAGACGGTCACTC	46
Primer 1	XGGTCTTGTTTCTTCTCTGTG	165
Primer 2	ATATATATGAGTGACCGTCTGCCTG	47
Primer 3	AB'AB'TTTTTTTTGGTCTTGTTTCTTCTCTGTG	48
Primer 4	TTTTTTTTGGTCTTGTTTCTTCTCTGTG	169

[00125] B. Preparation of Target Protein

[00126] Untagged human β-NGF (R&D Systems) was biotinylated by covalent coupling of NHS-PEO4-biotin (Pierce) to lysines residues. Protein (300 pmol in 50 µL) was exchanged into SB17T with a Sephadex G-25 microspin column. NHS-PEO4-biotin was added to 1.5 mM and the reaction was incubated at 4°C for 16 hours. Unreacted NHS-PEO4-biotin was removed with a Sephadex G-25 microspin column.

[00127] C. Immobilization of Target Protein

[00128] Target protein was immobilized on MyOne-SA paramagnetic beads (MyOne SA, Invitrogen, or hereinafter referred to as SA beads) for Round 1 of SELEX. β-NGF was diluted to 0.2 mg/mL in 0.5 mL SB17T and added to 0.5 mL SA beads (pre-washed twice with 20 mM NaOH and once with SB17T). The mixture was rotated for 30 minutes at 25°C and stored at 4°C until use.

[00129] D. Aptamer Selection with Slow Off-Rate Enrichment Process and Photocrosslinking

[00130] Selections were performed with the candidate mixture, comparing binding between samples with target protein (signal S) and samples without target protein (background B). The first three rounds were performed with selection for affinity (no photocrosslinking); the

second and third included slow off-rate enrichment process. Rounds four through nine included both slow off-rate enrichment process and photocrosslinking.

[00131] For each sample, a 90 µL DNA mixture was prepared in SB17T with 10-20 pmoles candidate mixture (56 pmoles in the first round) and 56 pmoles reverse primer. Samples were heated to 95°C for 3 minutes and cooled to 37°C at a rate of 0.1C/second. Samples were combined with 10 µL protein competitor mixture (0.1% HSA, 10 µM casein, and 10 µM prothrombin in SB17T), added to 0.5 mg SA beads and incubated at 37°C for 5 minutes with mixing. Beads were removed by magnetic separation.

[00132] Binding reactions were performed by adding 10 µL target protein (0.5 µM in SB17T) or SB17T to 40 µL DNA mixtures and incubating at 37°C for 30 min. The slow-off rate enrichment process was employed in three different ways. In rounds two and three, samples were diluted 20-fold by adding 950 µL SB17T (preheated to 37°C), and incubated at 37°C for 15 minutes prior to capturing complexes. In rounds four and five, samples were diluted 20-fold by adding 950 µL SB17T (preheated to 37°C), and incubated at 37°C for 30 minutes prior to crosslinking. In rounds six and seven, samples were diluted 20-fold by adding 950 µL SB17T (preheated to 37°C). 50 µL of each diluted sample was diluted again by transferring to 950 µL SB17T containing 10 mM dextran sulfate (5 kDa) (preheated to 37°C) to give an overall 400-fold dilution, and incubated at 37°C for 60 minutes prior to crosslinking. In rounds eight and nine, samples were diluted 20-fold by adding 950 µL SB17T (preheated to 37°C), and 50 µL of each sample was diluted again by transferring to 950 µL SB17T (preheated to 37°C) to give 400-fold dilution. Finally, 50 µL of each 400-fold diluted sample was diluted again by transferring to 950 µL SB17T containing 10 mM dextran sulfate (5 kDa) (preheated to 37°C) to give an overall 8000-fold dilution, and incubated at 37°C for 60 minutes prior to crosslinking. When photo-crosslinking was employed, the 1 mL binding reactions after the slow off-rate enrichment process were irradiated from above with an array of 470 nm LEDs for 60 seconds prior to complex capture.

[00133] Complexes were captured on SA beads via protein biotins by adding 0.25 mg MyOne-SA beads (Invitrogen) and incubating at 25°C for 15 minutes with mixing. Free DNA was removed by washing the beads five times with SB17T. Unless indicated, all washes were

performed by resuspending the beads in 100 µL wash solution, mixing for 30 seconds at 25°C, separating the beads with a magnet, and removing the wash solution. The aptamer strand was eluted from the beads by adding 85 µL 20 mM NaOH, and incubating at 37°C for 1 minute with mixing. 80 µL aptamer eluate was transferred to a new tube after magnetic separation, neutralized with 20 µL 80 mM HCl, and buffered with 1 µL 0.5 M Tris-HCl, pH 7.5.

[00134] When photo-selection was employed complexes were captured as above, and non-crosslinked DNA was removed by washing the beads once with 4 M guanidine-HCl containing 0.05% TWEEN-20 at 50°C for 10 minutes, once with 20 mM NaOH at 25°C for 2 minutes, twice with SB17T, and once with 16 mM NaCl. Crosslinked DNA was not removed from the bead surface for the amplification steps.

[00135] **E. Aptamer Amplification and Purification**

[00136] Selected aptamer DNA was amplified and quantified by QPCR. 48 µL DNA was added to 12 µL QPCR Mix (5X KOD DNA Polymerase Buffer, 25 mM $MgCl_2$, 10 µM forward PCR primer (Primer 2, SEQ ID NO: 47), 10 µM biotinylated reverse PCR primer (Primer 3, SEQ ID NO: 48), 5X SYBR Green I, 0.125 U/ µL KOD XL DNA Polymerase, and 1 mM each dATP, dCTP, dGTP, and dTTP) and thermal cycled in a Bio-Rad MyIQ QPCR instrument with the following protocol: 1 cycle of 99.9°C, 15 sec, 55°C, 10 sec, 68°C, 30 min, 30 cycles of 99.9°C, 15 seconds, 72°C, 1 minute. Quantification was done with the instrument software and the number of copies of DNA selected, with and without target protein, was compared to determine signal/background ratios.

[00137] When photo-selection was employed, a cDNA copy of the selected DNA was prepared by primer extension on the bead surface. Washed beads were resuspended in 20 µL cDNA extension mix (Primer Extension Buffer containing 5 µM non-biotinylated reverse PCR primer (Primer 4, SEQ ID NO: 169), 0.5 mM each dATP, dCTP, dGTP, and dTTP, and 0.125 U/ µL KOD XL DNA Polymerase) and incubated at 68°C for 30 minutes with mixing. The beads were washed 3 times with SB17T, and the cDNA strand was eluted from the beads by adding 85 µL 20 mM NaOH, and incubating at 37°C for 1 minute with mixing. 80 µL aptamer eluate was transferred to a new tube after magnetic separation, neutralized with 20 µL 80 mM HCl, and

buffered with 1 µL 0.5 M Tris-HCl, pH 7.5. The cDNA was amplified and quantified by QPCR as above with 30 cycles of 99.9°C, 15 seconds, 72°C, 1 minute.

[00138] Following amplification, the PCR product was captured on SA beads via the biotinylated antisense strand. 1.25 mL SA beads (10 mg/mL) were washed twice with 0.5 mL 20 mM NaOH, once with 0.5 mL SB17T, resuspended in 1.25 mL 3 M NaCl + 0.05% Tween, and stored at 4°C. 25 µL SA beads (10 mg/mL in 3 M NaClT) were added to 50 µL double-stranded QPCR products and incubated at 25°C for 5 minutes with mixing. The beads were washed once with SB17T, and the "sense" strand was eluted from the beads by adding 200 µL 20 mM NaOH, and incubating at 37°C for 1 minute with mixing. The eluted strand was discarded and the beads were washed 3 times with SB17T and once with 16 mM NaCl.

[00139] Aptamer sense strand was prepared with the ANA chromophore by primer extension from the immobilized antisense strand. The beads were resuspended in 20 µL primer extension reaction mixture (1X Primer Extension Buffer, 1.5 mM $MgCl_2$, 5 µM forward primer with 5' ANA chromophore (Primer 1, SEQ ID NO: 165), 0.5 mM each dATP, dCTP, dGTP, and BndUTP, and 0.125 U/µL KOD XL DNA Polymerase) and incubated at 68°C for 30 minutes with mixing. The beads were washed 3 times with SB17T, and the aptamer strand was eluted from the beads by adding 85 µL 20 mM NaOH, and incubating at 37°C for 1 minute with mixing. 80 µL aptamer eluate was transferred to a new tube after magnetic separation, neutralized with 20 µL 80 mM HCl, and buffered with 5 µL 0.1 M HEPES, pH 7.5.

[00140] **F. Selection Stringency and Feedback**

[00141] The relative target protein concentration of the selection step was lowered each round in response to the S/B ratio as follows, where signal S and background B are defined in Section D above:

$$\text{If } S/B < 10,\ [P]_{(i+1)} = [P]_i$$

$$\text{If } 10 \leq S/B < 100,\ [P]_{(i+1)} = [P]_i / 3.2$$

$$\text{If } S/B \geq 100,\ [P]_{(i+1)} = [P]_i / 10$$

where [P] = protein concentration and i = current round number.

[00142] After each selection round, the convergence state of the enriched DNA mixture was determined. 10 µL double-stranded QPCR product was diluted to 200 µL with 4 mM $MgCl_2$ containing 1X SYBR Green I. Samples were overlaid with 75 µL of silicon oil and

analyzed for convergence using a C_0t analysis which measures the hybridization time for complex mixtures of double stranded oligonucleotides. Samples were thermal cycled with the following protocol: 3 cycles of 98°C, 1 minute, 85°C, 1 minute; 1 cycle of 93°C, 1 minute, 85°C, 15 minutes. During the 15 minutes at 85°C, fluorescent images were measured at 5-second intervals. The fluorescence intensity was plotted as a function of log (time), and an increased rate of hybridization with each SELEX round was observed, indicating sequence convergence.

[00143] G. Clone Screening Process & Aptamer Identification

[00144] The converged pool after nine rounds of SELEX was cloned and sequenced. Selected DNA was PCR amplified with non-biotinylated SELEX primers to create AGCT DNA, purified using a QIAquick 96 PCR Purification Kit (Cat#28181), and purified inserts were cloned using Stratagene PCR-Script Cloning Kit (Cat#211189) as per manufacturer's protocol. The ligated SELEX pools were sent to a sequencing vender (Cogenics, Houston, Texas) for transformation, array into 96-well plates, DNA prep and sequencing. Sequences for ~42 clones were obtained and analyzed for convergence using custom software that determines sequence counts/copy number and identifies common convergence patterns using a local-alignment algorithm. Sequences with highest representation/copy number in the pool and sequences that were converged to common binding motifs were chosen for downstream screening. Six sequences were chosen for further analysis and were prepared enzymatically using plasmid DNA obtained from Cogenics as template for PCR amplification.

[00145] H. Measurement of Equilibrium Binding Constant (K_d)

[00146] The equilibrium binding constants of the 6 chosen sequences were measured in an affinity assay. Radiolabeled DNA was heated for 3 minutes at 95°C in SB17T-0.002 (SB17T with TWEEN-20 reduced to 0.002%) and slowly cooled to 37°C. Complexes were formed by mixing a low concentration of radiolabeled DNA (~$1x10^{-11}$ M) with a range of concentrations of target protein ($1x10^{-7}$ M to $1x10^{-12}$ M) in SB17T-0.002, and incubating at 37°C for 30 minutes. A portion of each reaction was transferred to a nylon membrane and dried to determine total counts in each reaction. Complexes were captured on ZORBAX resin (Agilent), passed through a MultiScreen HV Plate (Millipore) under vacuum, and washed with 200 µL SB17T-0.002 Buffer to separate protein-bound complexes from unbound DNA. The nylon membrane and

MultiScreen HV Plate were phosphorimaged and the amount of radioactivity in each sample quantified using a FUJI FLA-3000. The fraction of captured DNA was plotted as a function of protein concentration (P_t) and equilibrium binding constants (K_d) were determined using y = (max – min)(P_t)/(K_d + P_t) + min. Clone 2426-66 (SEQ ID NO: 1, listed in Table 3), a 76-mer, with a K_d = 5 x 10^{-9} M was selected as the lead clone for further characterization, see Figure 1.

[00147] **I. Deep Sequencing of SELEX Pool**

[00148] To evaluate more completely the sequences within the 2426-66 aptamer family, the enriched pool was sequenced using 454 pyrosequencing technology. The pool DNA was amplified with 454 primers as described above and the PCR product was purified and normalized using a Sequal normalization plate (Invitrogen, Cat# A10510-01). The eluate was run on a gel to confirm the size and purity of each amplicon. The purified PCR product was sequenced at the 454 pyrosequencing facility at the University of Colorado Health Science Center in Aurora CO.

[00149] The 454 sequences were aligned with 2426-66 by CLUSTAL analysis. From the total of 1165 multi-copy sequences, 165 sequences had similar pattern to 2426-66. Based on 5' sequence commonalities in these sequences, they were aligned into three groups. The middle region of sequence was conserved in all three groups. For all the sequences, the percentage identity at each position with 2426-66 was calculated as listed in Figure 2B. Table 2 lists a number of sequences representative of the 2426-66 aptamer family of sequences, wherein Z' represents a BndU.

Table 2. Sequences Representative of the 2426-66 Aptamer*

Aptamer Designation	Sequence (5'→3')	SEQ ID NO:
2426-87	CAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'GGCCGZ'Z'GZ'GG	49
2426-88	CAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'GC	50
2426-1373	GCAGCGGGACACAZ'GAGGACAZ'GGGGZ'Z'Z'AGCCGZ'Z'GZ'GG	51
2426-1621	GCAGCGGGACACAZ'GAGGACCZ'GGGGZ'Z'Z'AGCCGZ'Z'GZ'GG	52
2426-1634	GCGGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'GG	53
2426-1627	GCAGCGGGACACAZ'Z'AGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'GG	54

2426-1372	GCAGCGGAACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'GG	55
2426-1387	GCAGCGGZ'ACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'GG	56
2426-1527	CAGCGGGACACAZ'GAGGACZ'Z'GGGZ'Z'Z'AGCCGZ'Z'GZ'GGCA	57
2426-1753	Z'CAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'GG	58
2426-1003	GCAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'GG	59
2426-1626	GCAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGZ'CGZ'Z'GZ'GG	60
2426-1380	GCAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCAZ'Z'GZ'GG	61
2426-1625	GCAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCZ'Z'Z'GZ'GG	62
2426-1388	GCAGCGZ'GACAZ'AZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'GG	63
2426-1381	GCAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z' GZ'AG	64
2426-1699	GZ'AGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AACCGZ'Z'GZ'GG	65
2426-1702	GZ'AGCGGGACACAZ'GGGGACZ'Z'GGGGZ'Z'Z'AACCGZ'Z'GZ'GG	66
2426-1265	GAAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AACCGZ'Z'GZ'GG	67
2426-1374	GCAGCGGGACACAZ'GAGGACZ'Z'GAGGZ'Z'Z'AACCGZ'Z'GZ'GG	68
2426-1377	GCAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AACCGZ'Z'GZ'GG	69
2426-1384	GCAGCGGGACACAZ'GAGZ'ACZ'Z'GGGGZ'Z'AACCGZ'Z'GZ'GGC	70
2426-1622	GCAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'AACCGZ'Z'GZ'GGC	71
2426-1378	GCAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AACCGZ'Z'GZ'GZ'	72
2426-1266	GAAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'Z'G	73
2426-1537	GAAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'GG	74
2426-1355	GCAACGGGACACAZ'GAZ'GACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'GG	75
2426-1385	GCAGCGGGACACAZ'GAZ'GACZ'Z'Z'GGGZ'Z'Z'AGCCGZ'Z'AZ'GG	76

2426-1701	GZ'AGCGGGACACAZ'GAZ'GACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'GG	77
2426-1458	GZ'AGCGGGACACAZ'GAGGACZ'Z'GGGGGZ'Z'AGCCGZ'Z'GCGG	78
2426-1700	GZ'AGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'GG	79
2426-1386	GCAGCGGGGCACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'GG	80
2426-1623	GCAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCAGZ'Z'AZ'GG	81
2426-1392	GCAGZ'GGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'AZ'GC	82
2426-1624	GCAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'AZ'GC	83
2426-1383	GCAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'GC	84
2426-1382	GCAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'GA	85
2426-1619	GCAGCCGGACACAZ'GAGZ'ACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'GG	86
2426-1389	GCAGCZ'GGACACACGAGGACZ'Z'GGGGZ'Z'Z'AGCZ'GZ'Z'GZ'GG	87
2426-1371	GCAGCAGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'GG	88
2426-1391	GCAGZ'AGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'GG	89
2426-1618	GCAGAGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'GG	90
2426-1629	GCAGZ'GGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'GG	91
2426-1393	GCAGZ'GGGAZ'ACAZ'GAGGACGZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'G	92
2426-1457	GZ'AGCGGGACACAZ'GAGGACGZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'G	93
2426-1642	GGAGCGGGGCACAZ'GAGGACZ'Z'GGGGGZ'Z'Z'AGCCGZ'Z'GZ'G	94
9999-1044	GCAGCGGGACACAZ'GAGGACZ'Z'GGGGGZ'Z'Z'AGCCGZ'Z'GZ'G	95
2426-1628	GCAGCGGGACACZ'AZ'GAGGACZ'Z'GGGGZ'Z'Z'AGGCCGZ'Z'GZ'	96
2426-1376	GCAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AACCGZ'Z'	97
2426-1379	GCAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AG	98

2426-1375	GCAGCGGGACACAZ'GAGGACZ'Z'GGGGGZ'Z'Z'AGC	99
2426-1390	GCAGCZ'GGACACAZ'GAZ'GZ'ACGZ'Z'GGGGZ'Z'Z'AGCC	100
2426-1390	GCAGCZ'GGACACAZ'GAZ'GZ'ACGZ'Z'GGGGZ'Z'Z'AGCC	101
2426-1402	GGAACZ'AGCGZ'GGAZ'GGGGCZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'AZ'GC	102
2426-1531	GAACZ'AGCGZ'GGAZ'GGGGGCZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'AZ'GC	103
2426-1401	GGAACZ'AGCGZ'GAZ'GGGGGCZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'AZ'GC	104
2426-1755	Z'GGAACZ'AGCGZ'GGAZ'GGGGGCZ'Z'GGGZ'Z'Z'AGCCG Z'Z'AZ'G	105
2426-1404	GGAACZ'AGCGZ'GGAZ'GGGGGCZ'Z'GGGGZ'Z'Z'AGCCAZ'Z'AZ'G	106
2426-1009	GGAACZ'AGCGZ'GGAZ'GGGGGCZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'AZ'G	107
2426-1403	GGAACZ'AGCGZ'GGAZ'GGGGGCZ'Z'GGAGZ'Z'Z'AGCCGZ'Z'AZ'G	108
2426-1637	GGAACZ'AGCGZ'GGAZ'GGGGGCZ'Z'GGGGZ'Z'Z'AACCGZ'Z'AZ'G	109
2426-1529	GAAACZ'AGCGZ'GGAZ'GGGGGCZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'AZ'G	110
2426-1638	GGAACZ'AGCGZ'GGAZ'GGGGGCZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'G	111
2426-1643	GGAGCZ'AGCGZ'GGAZ'GGGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'G	112
2426-1636	GGAACZ'AGCACGGAZ'GGGGGCZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'AZ'G	113
2426-1405	GGAACZ'AGCGZ'GGAZ'GGGGGCZ'Z'GGGGZ'Z'Z'AZ'CCGZ'Z'AZ'A	114
2426-1406	GGAACZ'AGCGZ'GGAZ'GGGGGCZ'Z'GGGZ'Z'AGZ'	115
2426-1364	GCAGAAZ'GCGGZ'AZ'AZ'GAGGACZ'Z'GGAGZ'Z'Z'AGCCGZ'Z'GZ'	116
2426-1365	GCAGAAZ'GCGGZ'AZ'AZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'	117
2426-1366	GCAGAAZ'GCGGZ'AZ'AZ'GGGGACZ'Z'GGGGZ'Z'Z'GGCCGZ'Z'GZ'	118
2426-1617	GCAGAAZ'GCGGZ'AZ'AZ'GGGGGCZ'Z'GGGGZ'Z'Z'AZ'CCGZ' Z'GZ'	119
2426-1367	GCAGAAZ'GCGGZ'AZ'AZ'GGGGGCZ'Z'GGGGZ'Z'Z'AGCCAZ'Z'GZ'	120

2426-1067	GCAGAAZ'GAGGZ'AZ'AZ'GAGGACZ'Z'GGG GZ'Z'Z'AGCCGZ'Z'GZ'	121
2426-1369	GCAGAAZ'GCGGZ'AZ'AZ'GGGGGCZ'Z'GGG GZ'Z'Z'AZ'CCGZ'Z'AZ'	122
2426-1368	GCAGAAZ'GCGGZ'AZ'AZ'GGGGGCZ'Z'GGG GZ'Z'Z'AGCCGZ'Z'AZ'	123
2426-1370	GCAGAAZ'GCGGZ'AZ'AZ'GGGGGCZ'Z'Z'GG GZ'Z'Z'AGCCGZ'Z'AZ'	124
2426-1616	GCAGAAZ'GCGGZ'AZ'AZ'GGGGACZ'Z'GGG GGZ'Z'Z'AGCCGZ'Z'G	125
2426-1363	GCAGAAZ'GCGGZ'AZ'AGZ'GGGGGCZ'Z'GG GGZ'Z'Z'AGCCGZ'Z'A	126
2426-1519	Z'GCAGAAZ'GCGGZ'AZ'AZ'GGGGGCZ'Z'GG GGZ'Z'Z'AZ'CCG	127
2426-1156	GZ'GZ'CACZ'Z'GZ'GGGGAGZ'Z'GGGGZ'Z'GA Z'CCGZ'Z'GZ'CCGCC	128
2426-1743	GZ'GZ'CACZ'Z'GZ'GGGGAGZ'Z'GGGGZ'Z'GA Z'CCGZ'Z'GZ'Z'CGCC	129
2426-1514	GZ'GZ'CACZ'Z'GZ'GGGGAGZ'Z'GGGGZ'Z'GA Z'CCGZ'Z'GZ'Z'Z'CGZ'	130
2426-1513	GZ'GZ'CACZ'CGZ'GGGGAGZ'Z'GGGGZ'Z'GA Z'CCGZ'Z'GZ'Z'CGCZ'	131
2426-1742	GZ'GZ'CACZ'Z'GZ'GGGGAGZ'Z'GGGGZ'Z'GA Z'CCAAZ'GZ'Z'CGCZ'	132
2426-1744	GZ'GZ'CACZ'Z'GZ'GGGGAGZ'Z'GGGGZ'Z'GA Z'CCGZ'Z'GZ'Z'Z'CGC	133
2426-1157	GZ'GZ'CACZ'Z'GZ'GGGGAGZ'Z'Z'GGGZ'Z'GZ 'AZ'CCGZ'Z'CGZ'Z'Z'C	134
2426-1094	GGCGACGCGCACAGZ'GGGGZ'AGZ'Z'GGG GZ'Z'Z'AACCGZ'Z'GZ'	135
2426-1417	GGCGACGCGCGCAZ'AGGGZ'AGZ'Z'GGGG Z'Z'Z'AACCGZ'Z'GZ'C	136
2426-63	GCGACGCGCGCAZ'GGGGZ'AGZ'Z'GGGGZ' Z'Z'AACGGZ'Z'GZ'CG	137
2426-1038	GACCAACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGC CGZ'Z'GZ'GGCACAG	138
2426-1571	GACCAACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGC CGZ'Z'GZ'Z'GCACAG	139
2426-1100	GGGAAGCGAZ'AZ'GAGGACZ'Z'GGGG Z'Z'Z'AGCCGZ'Z'GZ'GGCA	140
2426-1419	GGGAAGCGAZ'AZ'GAGGAG Z'Z'GGGGZ'Z'Z'AZ'CCGZ'Z'GZ'CAAC	141
2426-1089	GGAGZ'AGGGAAAAZ'GGGGAGZ'Z'GGGGZ' Z'Z'AZ'CCGZ'Z'GZ'CA	142

2426-1064	GAZ'Z'GCZ'GGA GGA Z'GG GGAGZ'GGGG G Z'Z'Z'AZ' CCGZ'Z' GZ'CA	143
2426-1352	GAZ'Z'GCZ'GGAGGAZ'GGGGAGZ'Z'GGGGZ' Z'Z'AZ'CCGZ'Z'GZ'CA	144
2426-1198	GAZ'Z'GCZ'GGAGGAZ'GAGGACZ'Z'GGGGZ' Z'Z'AGCCGZ'Z'GZ'GG	145
2426-1073	GCCGGGGCCGCZ'AZ'GAGGACAZ'GGGGZ'Z 'Z'AGCCGZ'Z'GZ'GG	146
2426-1068	GCAGAAZ'GCGAZ'AZ'AZ'GGGGGCZ'Z'GGG GZ'Z'Z'AGCCGZ'Z'AZ'	147
2426-1231	GGZ'GGCACACZ'GGZ'GGGGGGCZ'Z'GGGG Z'Z'GAGCCGZ'Z'AZ'G	148

* Only the 40N region is shown

[00150] Based on this, as noted above, the consensus sequence for binding to β-NGF was determined to be the following sequence:

BAZGRGGRSZZGGGGZZZADCCGZZRZG (SEQ ID NO: 3)

wherein B, Z, R, and S are as defined above. While the consensus sequence is 28 nucleotides in length, a number of the sequences listed in Table 2 had single-base insertions in this consensus. Figure 2B indicates approximately 91 percent of the sequences had a deletion at position 12, but approximately 7 percent of the sequences had a G or Z at this position, and approximately 95 percent had a deletion at position 19, but approximately 3 percent had a G at this position. This observation suggests insertions in the β-NGF consensus sequence (SEQ ID NO: 3) are tolerated at some positions and these insertions will not inactivate the β-NGF aptamer.

Example 2. Sequence Truncation Studies

[00151] β-NGF aptamer 2426-66 (SEQ ID NO: 1) is 76 nucleotides in length with a K_d = 5 x 10^{-9} M. For most efficient chemical synthesis, it is important to identify the minimal high-affinity aptamer sequence and truncate the aptamer to the smallest size possible. Other advantages are increased tissue penetration and stability against nuclease activity *in vivo*.

[00152] In order to identify the minimal region of aptamer 2426-66 (SEQ ID NO: 1) that retains binding affinity, a series of truncated variants were synthesized representing all possible contiguous 50 nucleotide long sequences present in 2426-66. Sequences of the variants are listed

in Table 3, wherein Z' represents BndU and T represents dT. The variants were tested for affinity to β-NGF in the affinity binding assay as described above.

Table 3. Sequences of Aptamer 2426-66 and Truncated Variants

Aptamer Designation	Sequence (5'→ 3')	SEQ ID NO:
2426-66 (76-mer)	GAGTGACCGTCTGCCTGCAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'GGCACAGAGAAGAAACAAGACC	1
2426-66-2 (50-mer)	GAGTGACCGTCTGCCTGCAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCG	4
2426-66-3 (50-mer)	AGTGACCGTCTGCCTGCAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'	5
2426-66-4 (50-mer)	GTGACCGTCTGCCTGCAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'	6
2426-66-5 (50-mer)	TGACCGTCTGCCTGCAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'G	7
2426-66-6 (50-mer)	GACCGTCTGCCTGCAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GT	8
2426-66-7 (50-mer)	ACCGTCTGCCTGCAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'G	9
2426-66-8 (50-mer)	CCGTCTGCCTGCAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'GG	10
2426-66-9 (50-mer)	CGTCTGCCTGCAGCGGGACACAZ'Z''GAGGACZ'Z''Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'GGC	11
2426-66-10 (50-mer)	GTCTGCCTGCAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'GGCA	12
2426-66-11 (50-mer)	TCTGCCTGCAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'GGCAC	13
2426-66-12 (50-mer)	CTGCCTGCAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'GGCACA	14
2426-66-13 (50-mer)	TGCCTGCAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'GGCACAG	15
2426-66-14 (50-mer)	GCCTGCAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'GGCACAGA	16
2426-66-15 (50-mer)	CCTGCAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'GGCACAGAG	17
2426-66-16 (50-mer)	CTGCAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'GGCACAGAGA	18
2426-66-17 (50-mer)	TGCAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'GGCACAGAGAA	19
2426-66-18 (50-mer)	GCAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'GGCACAGAGAAG	20

2426-66-19 (50-mer)	CAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AG CCGZ'Z'GZ'GGCACAGAGAAGA	21
2426-66-20 (50-mer)	AGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGC CGZ'Z'GZ'GGCACAGAGAAGAA	22
2426-66-21 (50-mer)	GCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCC GZ'Z'GZ'GGCACAGAGAAGAAA	23
2426-66-22 (50-mer)	CGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCG Z'Z'GZ'GGCACAGAGAAGAAAC	24
2426-66-23 (50-mer)	GGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ' Z'GZ'GGCACAGAGAAGAAACA	25
2426-66-24 (50-mer)	GGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z' GZ'GGCACAGAGAAGAAACAA	26
2426-66-25 (50-mer)	GACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'G Z'GGCACAGAGAAGAAACAAG	27
2426-66-26 (50-mer)	ACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ' GGCACAGAGAAGAAACAAGA	28
2426-66-38 (50-mer)	CACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'G GCACAGAGAAGAAACAAGAC	29
2426-66-39 (50-mer)	ACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'GG CACAGAGAAGAAACAAGACC	30

[00153] All variants retained β-NGF binding activity with the exception of variants 2426-66-2 (SEQ ID NO: 4), 2426-66-3 (SEQ ID NO: 5), 2426-66-4 (SEQ ID NO: 6), 2426-66-5 (SEQ ID NO: 7), and 2426-66-6 SEQ ID NO: 8), suggesting the 5' terminal 26 nucleotides (positions 1-26) and 3' terminal 21 nucleotides (positions 56-76) of 2426-66 were not required for binding β-NGF, and all or part of the remaining 29 nucleotide element (positions 27-55) may be sufficient. This hypothesis was tested by synthesizing and measuring binding affinities of a second series of variants of 2426-66 (SEQ ID NO: 1). Sequences of the variants are listed in Table 4, wherein Z' represents BndU and T represents dT. All variants retained β-NGF binding activity with the exception of variants 2426-66-56 (SEQ ID NO: 150), 2426-66-57 (SEQ ID NO: 151), 2426-66-58 (SEQ ID NO: 152), and 2426-66-59 (SEQ ID NO: 153). Variant 2426-66-55 (SEQ ID NO: 149), a 25mer, was the shortest sequence with β-NGF binding affinity equal to the full-length aptamer 2426-66 (SEQ ID NO: 1). Variant 2426-66-54 (SEQ ID NO: 44), a 26mer, had affinity for β-NGF slightly better than the full length aptamer 2426-66 (SEQ ID NO: 1) and was chosen for further optimization.

Table 4. Sequences of Truncated Variants of Aptamer 2426-66

Aptamer Designation	Sequence (5'→ 3')	SEQ ID NO:
2426-66-30 (40-mer)	TGCAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGC CGZ'Z'GZ'G	31
2426-66-40 (39-mer)	GCAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGC CGZ'Z'GZ'G	32
2426-66-41 (38-mer)	CAGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCC GZ'Z'GZ'G	33
2426-66-42 (37-mer)	AGCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ 'Z'GZ'G	34
2426-66-52 (36-mer)	GCGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ' Z'GZ'G	35
2426-66-43 (35-mer)	CGGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z' GZ'G	36
2426-66-44 (34-mer)	GGGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'G Z'G	37
2426-66-45 (33-mer)	GGACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ' G	38
2426-66-46 (32-mer)	GACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'G	39
2426-66-47 (31-mer)	ACACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'G	40
2426-66-48 (30-mer)	CACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'G	41
2426-66-49 (29-mer)	ACAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'G	42
2426-66-50 (28-mer)	CAZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'G	2
2426-66-53 (27-mer)	AZ'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'G	43
2426-66-54 (26-mer)	Z'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'G	44
2426-66-55 (25-mer)	GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'G	149
2426-66-56 (24-mer)	AGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'G	150
2426-66-57 (23-mer)	GGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'G	151
2426-66-58 (22-mer)	GACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'G	152
2426-66-59 (21-mer)	ACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'G	153

Example 3. Determination of Desirable BndU positions

[00154] 26-mer single substitution of BndU to dT

[00155] Nine of the 26 nucleotides of aptamer 2426-66-54 (SEQ ID NO: 44) are BndU. To determine which BndU positions are involved in binding, nine variants of 2426-66-54 were synthesized, each containing a single dT substitution at one of the nine BndU positions and β-NGF affinities were measured. Sequences of the variants are listed in Table 5 wherein Z' represents BndU and T represents dT.

Table 5. Sequences of Variants of Aptamer 2426-66-54

Aptamer Designation	Substitution Position	Sequence (5'→ 3')	SEQ ID NO:
2426-66-54	none	Z'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'G	44
2426-66-66	1	**T**GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'G	156
2426-66-67	8	Z'GAGGAC**T**Z'GGGGZ'Z'Z'AGCCGZ'Z'GZ'G	157
2426-66-68	9	Z'GAGGACZ'**T**GGGGZ'Z'Z'AGCCGZ'Z'GZ'G	158
2426-66-69	14	Z'GAGGACZ'Z'GGGG**T**Z'Z'AGCCGZ'Z'GZ'G	159
2426-66-70	15	Z'GAGGACZ'Z'GGGGZ'**T**Z'AGCCGZ'Z'GZ'G	160
2426-66-71	16	Z'GAGGACZ'Z'GGGGZ'Z'**T**AGCCGZ'Z'GZ'G	161
2426-66-72	22	Z'GAGGACZ'Z'GGGGZ'Z'Z'AGCCG**T**Z'GZ'G	162
2426-66-73	23	Z'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'**T**GZ'G	163
2426-66-74	25	Z'GAGGACZ'Z'GGGGZ'Z'Z'AGCCGZ'Z'G**T**G	164

[00156] Substitution of BndU with dT at position 9 (variant 2426-66-68, SEQ ID NO: 158) and position 16 (variant 2426-66-71, SEQ ID NO: 161) showed no loss of affinity for β-NGF compared with the unsubstituted (all BndU) aptamer 2426-66-54 (SEQ ID NO: 44). Substitution of BndU with dT at position 1 (variant 2426-66-66, SEQ ID NO: 156), position 8 (variant 2426-66-67, SEQ ID NO: 157) and position 14 (2426-66-69, SEQ ID NO: 159) showed partial loss of affinity, and substitution at position 15 (2426-66-70, SEQ ID NO: 160), position

22 (2426-66-72, SEQ ID NO: 162), position 23 (2426-66-73, SEQ ID NO: 163) and position 25 (2426-66-74, SEQ ID NO: 164) showed complete loss of affinity. These results indicate that modified uridine residues at positions 1, 8, 14, 15, 22, 23 and 25 are desirable for maximal β-NGF binding affinity

[00157] Truncated variants of 2426-66 (SEQ ID NO: 1) with BndU residues at positions 9 and 16 replaced with dT were synthesized and tested for affinity to β-NGF. Sequences are listed in Table 6 wherein Z' represents a BndU and T represents dT. Substitution of BndU with dT at two positions showed no loss of affinity compared to unsubstituted controls for any of the three variants.

Table 6. Truncated and Substituted Variants of Aptamer 2426-66

Aptamer Designation	**Substitution Positions**	**Sequence (5'→3')**	**SEQ ID NO:**
2426-66-75 (28mer)	11, 18	CAZ'GAGGACZ'**T**GGGGZ'Z'**T**AGCCGZ'Z'GZ'G	166
2426-66-76 (27mer)	10, 17	AZ'GAGGACZ'**T**GGGGZ'Z'**T**AGCCGZ'Z'GZ'G	167
2426-66-77 (26mer)	9, 16	Z'GAGGACZ'**T**GGGGZ'Z'**T**AGCCGZ'Z'GZ'G	168

Based on these results, which are summarized in Figure 2A, the consensus sequence (SEQ ID NO: 3) for binding to β-NGF was modified as follows to reflect the ability to substitute dT for BndU at two positions:

BAZGRGGRSZ**W**GGGGZZ**W**ADCCGZZRZG (SEQ ID NO: 45)

wherein Z, R, S, Z and W are as defined above.

Example 4. Cell Assays

[00158] β-NGF aptamer 2426-66 (SEQ ID NO: 1) and truncated variants of 2426-66 were screened for inhibition of β-NGF activity in two *in vitro* cell assays. PC12 cells (CRL-1721 from ATCC), a cancer cell line from a rat pheochromocytoma and model for neuronal differentiation, respond to β-NGF by induction of the neuronal phenotype. Two manifestations of this response are the phosphorylation of membrane-bound TrkA and the extension of neurites. Aptamers were tested for their ability to inhibit β-NGF-stimulated TrkA phosphorylation and neurite growth of PC12 cells.

[00159] **Neurite Growth Assays**

[00160] PC12 cells were plated sparsely in 60 mm dishes and allowed to attach to the plate overnight. After attachment, normal growth medium was replaced with low-serum medium (LSM), as PC12 cells do not differentiate in normal high-serum growth medium. β- NGF (100 ng/mL) and aptamer (100 nM) were pre-mixed in LSM and allowed to equilibrate for one hour, then added to the plates to final concentrations of 10 ng/mL β- NGF (0.38 nM) and 10 nM aptamer. The cells were allowed to incubate for three days, and on day three, the media, β-NGF, and aptamer were replaced as before. On day 5, images of the cells were captured with a phase-contrast microscope and neurite length was measured using the NeuronJ plugin for ImageJ (NIH program). Neurite length/cell was calculated and normalized to a value of 100 for the no-aptamer control sample (relative to neurite growth).

[00161] The ability of aptamers to inhibit human β-NGF induced differentiation of PC12 cells was tested in the Neurite Growth Assay. Aptamers were synthesized with an inverted dT amidite (3'-idT) on the 3' terminus to provide resistance to 3'- 5'exonucleases present in the culture medium (see Figure 5).

[00162] Aptamer 2426-66 (SEQ ID NO: 1) and truncated variant 2426-66-50 (SEQ ID NO: 2) effectively inhibited neurite growth induced with β-NGF. Variants 2426-66-53 (SEQ ID NO: 43), 2426-66-54 (SEQ ID NO: 44), and 2426-66-55 (SEQ ID NO: 149) were less effective at inhibiting β-NGF mediated neurite growth than 2426-66-50, indicating a minimum aptamer length of 28 nucleotides was required for maximal inhibition. Variant 2426-66-75 (SEQ ID NO: 166) in which BndU residues at positions 11 and 18 of 2426-66-50 were replaced by dT residues), was also not effective in blocking β-NGF mediated neurite growth. Variant 2426-66-3 (SEQ ID NO: 5), a 50-mer with poor affinity for β-NGF, showed little inhibition of β-NGF mediated neurite growth.

[00163] The effectiveness of aptamer inhibition of β-NGF mediated neurite growth was determined by measuring relative neurite growth at aptamer concentrations ranging from 0.5 nM to 8.0 nM and calculating the half maximal inhibitory concentration (IC_{50}) using a non-linear curve fit (sigmoidal dose response with variable slope) (see Figure 6). Aptamer 2426-66 (SEQ

ID NO: 1) exhibited an $IC_{50} = 2 \times 10^{-9}$ M, and truncated variant 2426-66-50 (SEQ ID NO: 2) exhibited an $IC_{50} = 1 \times 10^{-9}$ M in this assay.

[00164] Aptamer 2426-66 (SEQ ID NO: 1) and truncated variants 2426-66-50 (SEQ ID NO: 2) and 2426-66-53 (SEQ ID NO: 43) were tested for inhibition of mouse β- NGF and rat β-NGF in the Neurite Growth Assay. All three inhibited mouse β-NGF nearly as effectively as human β-NGF, and inhibited rat β-NGF to a lesser extent (see Figure 7).

[00165] **TrkA Phosphorylation Assay**

[00166] β-NGF binds to the TrkA receptor on the PC12 cell surface, inducing dimerization and auto-phosphorylation of the receptor. The TrkA Phosphorylation Assay examines the phosphorylation status of TrkA 10 minutes after treatment with β- NGF that has been pre-equilibrated with aptamer. While the Neurite Growth Assay is a terminal assay, looking at the end-point of β- NGF stimulation, the TrkA Phosphorylation Assay is a snapshot of the immediate signaling events following β- NGF stimulation.

[00167] PC12 cells were seeded on 100 mm plates and allowed to attach overnight. After attachment, the medium was changed to LSM. The cells were left in LSM overnight, and were then treated for 10 minutes with β-NGF alone (10 ng/mL final concentration, or 0.38 nM), β-NGF with TrkA phosphorylation inhibitor K252a (0.2 μM), and β- NGF pre-equilibrated with aptamer at 10 nM final concentration. Cells were collected, lysed, and TrkA was immuno-precipitated from the cleared lysate with a total Trk antibody (TrkA is the only Trk receptor expressed in PC12 cells). The immuno-precipitate was run on an SDS-PAGE gel, electro-blotted onto a PVDF membrane, and probed with a phospho-tyrosine antibody to quantify the amount of phosphorylated TrkA. The blot was stripped and probed with a TrkA antibody to quantify the amount of total TrkA. Percent TrkA phosphorylation (ratio of phosphorylated TrkA/total TrkA) was calculated for each sample and normalized to a value of 100 for the no-aptamer control. The results are set forth in Figure 8.

[00168] Aptamer 2424-66 (SEQ ID NO: 1) and truncated variants 2426-66-50 (SEQ ID NO: 2) and 2426-66-3 (SEQ ID NO: 5) were tested for inhibition of TrkA phosphorylation by human β-NGF. Aptamer 2426-66 and truncated variant 2426-66-50 effectively inhibited

phosphorylation of TrkA receptors induced with β- NGF. Variant 2426-66-3, a 50-mer with poor affinity for β-NGF, showed little inhibition of TrkA phosphorylation by β-NGF.

[00169] Variant 2426-66-50 (SEQ ID NO: 2) was tested for inhibition of mouse β- NGF and rat β-NGF in the TrkA phosphorylation Assay. The results are set forth in Figure 9. Variant 2426-66-50 effectively inhibited both mouse β- NGF and rat β-NGF induced TrkA phosphorylation.

Example 5. Aptamer Treatment of Atopic Dermatitis in Mouse Model System

[00170] Inbred NC/NgaTnd mice raised in non-sterile (conventional) circumstances spontaneously develop skin lesions similar to atopic dermatitis lesions in humans and are an established model for investigating treatments for atopic dermatitis (Matsuda *et al.*, Int. Immunol. 9:461, 1997). The following study was designed to assess the ability of NGF-neutralizing aptamers to reduce or eliminate the clinical manifestations of atopic dermatitis *in vivo* in this mouse model.

[00171] NC/NgaTnd mice were maintained in air-uncontrolled conventional circumstances at 20-26°C with a 12 hour day/night cycle, and given access to standard food and water *ad libitum*. Mice at the age of 8-10 weeks that manifested mild skin lesions (disease phenotype) were used in this study. NC/NgaTnd mice maintained under specific pathogen free (SPF) conditions and exhibiting no clinical signs or symptoms of atopic dermatitis (no disease phenotype) were used as controls.

[00172] Hydrophilic ointment (HO) was prepared according to the Japanese Pharmacopoeia (25% white petrolatum, 20% stearyl alcohol, 12% propylene glycol, 4% polyoxyethylene hydrogenated castor oil 60, 1% glyceryl monostearate, 0.1% methyl parahydroxybenzoate, 0.1% propyl parahydroxybenzoate). Aptamer was prepared in HO by melting 20 g of HO in a water bath at 85°C, adding 20 g of 2% aptamer in water, and mixing in an ice-cold water bath until cool.

[00173] Mice were divided into four groups. Group 1 contained 7 mice with dermatitis, untreated. Group 2 contained 7 mice with dermatitis, treated with HO. Group 3 contained 7 mice with dermatitis, treated with aptamer 2426-66-50 (SEQ ID NO: 2) with a 3'-idT (1% w/v in HO). Group 4 contained 7 normal SPF mice, untreated. Mice in groups 2 and 3 were treated

once daily for four weeks by applying 100 mg of sample to the affected dorsal areas. Once each week for four weeks (days 0, 7, 14, 21, 28) the scratching behavior and the clinical skin condition score of the mice were quantified.

[00174] Spontaneous scratching behavior was quantified using a SCLABA-Real system (Scratch Counting for LABoratory Animals, Noveltec Inc., Kobe, Japan) (Hattori *et al.*, J. Immunol. 184:2729, 2010). Mice were put into the SCLABA instrument 30 minutes before measurement to allow them to adapt to the new environment, and scratching number was counted for one hour in an observation chamber. A series of scratching behaviors, starting with stretching of the hind paws to the head, face, or back and ending with the set-back of the paws, was counted as one bout of scratching.

[00175] Clinical skin condition score was determined according to the criteria described in Matsuda *et al.*, Int. Immunol. 9:461, 1997. Observation items were 1) pruritus/itching, 2) erythema/hemorrhage, 3) edema, 4) excoriation/erosion, and 5) scaling/dryness. Scores for each observation item were graded as 0 (none), 1 (mild), 2 (moderate) and 3 (severe). The clinical skin condition score was the sum of the five observation item scores.

[00176] Figure 11 illustrates the change in scratching frequency for each treatment group, plotted as averages with standard error bars. Scratching frequency decreased steadily from day 14-28 for group 3 (aptamer treatment), but showed no change in frequency over 28 days for groups 1 (no treatment) and 2 (HO treatment). Scratching frequency of group 4 (normal SPF mice) was very low (data not shown). Figure 12 illustrates the change in clinical skin condition score for each treatment group, plotted as averages with standard error bars. Skin condition score decreased steadily from day 14-28 for group 3 (aptamer treatment), but showed no change over 28 days for groups 1 (no treatment) and 2 (HO treatment). Skin condition score of group 4 (normal SPF mice) was very low (data not shown).

[00177] The foregoing embodiments and examples are intended only as examples. No particular embodiment, example, or element of a particular embodiment or example is to be construed as a critical, required, or essential element or feature of any of the claims. Further, no element described herein is required for the practice of the appended claims unless expressly described as "essential" or "critical." Various alterations, modifications, substitutions, and other variations can be made to the disclosed embodiments without departing from the scope of the

present invention, which is defined by the appended claims. The specification, including the figures and examples, is to be regarded in an illustrative manner, rather than a restrictive one, and all such modifications and substitutions are intended to be included within the scope of the invention. Accordingly, the scope of the invention should be determined by the appended claims and their legal equivalents, rather than by the examples given above. For example, steps recited in any of the method or process claims may be executed in any feasible order and are not limited to an order presented in any of the embodiments, the examples, or the claims.

Table 7. Diseases with Potential for β-NGF			
AIDS dementia complex	Crohn's disease	Interstitial pneumonitis	Pruritis
Acquired immune deficiency syndrome (AIDS)	Cryptogenic autoimmune hepatitis	Ionizing radiation exposure	Psoriasis
Acquired immunodeficiency syndrome	Cryptogenic fibrosing alveolitis	Iridocyclitis/uveitis/optic neuritis	Psoriasis type 1
Acquired pernicious anemia	Culture negative sepsis	Irritable bowel syndrome	Psoriasis type 2
Acrocyanosis	Cystic fibrosis	Ischemia-reperfusion injury	Psoriatic arthritis
Acute and chronic pain (different forms of pain)	Cytokine therapy associated disorders	Ischemic stroke	Psoriatic arthropathy
Acute and chronic pain associated with infectious disease (bacterial or viral)	Deafferentation syndromes	Juvenile chronic arthritis	Pulmonary fibrosis
Acute immune disease associated with organ transplantation	Dementia pugilistica	Juvenile pernicious anemia	Pulmonary hypertension secondary to connective tissue disease
Acute leukemia	Demyelinating disease	Juvenile rheumatoid arthritis	Pulmonary manifestation of polyarteritis nodosa
Acute lymphoblastic leukemia (ALL)	Dengue hemorrhagic fever	Juvenile spinal muscular atrophy	Radiation fibrosis
Acute myeloid leukemia (AML)	Dental pain	Kaposi's sarcoma	Radiation therapy
Acute or chronic immune disease associated with organ transplantation	Dermatitis	Kawasaki's disease	Raynaud's phenomenon and disease
Acute pancreatitis	Dermatitis	Kidney transplant	Reactive arthritis

Table 7. Diseases with Potential for β-NGF			
	scleroderma	rejection	
Acute renal failure	Dermatologic conditions	Legionella	Refsum's disease
Acute rheumatic fever	Dermatological diseases	Leishmaniasis	Regular narrow QRS tachycardia
Acute transverse myelitis	Dermatomyositis / polymyositis associated lung disease	Leprosy	Reiter's disease
Addison's disease	Diabetes	Lesions of the corticospinal system	Renal disease NOS
Adenocarcinomas	Diabetes mellitus	Linear IgA disease	Renal diseases
Adult (acute) respiratory distress syndrome	Diabetic ateriosclerotic disease	Lipedema	Renovascular hypertension
Aerial ectopic beats	Diabetic neuropathy	Liver transplant rejection	Reperfusion injury
Alcohol-induced hepatitis	Diffuse Lewy body disease	Lupus	Reperfusion injury after organ transplantation
Alcohol-induced liver injury	Dilated cardiomyopathy	Lyme arthritis	Restrictive cardiomyopathy
Alcoholic cirrhosis	Dilated congestive cardiomyopathy	Lyme disease	Retinal Degeneration
Allergic and atopic diseases	Discoid lupus erythematosus	Lymphederma	Rett Syndrome
Allergic conjunctivitis	Disorders of the basal ganglia	Lymphocytic infiltrative lung disease	Rheumatic diseases
Allergic contact dermatitis	Disseminated intravascular coagulation	Malaria	Rheumatoid arthritis
Allergic diseases	Disturbances of visceral motility at respiratory	Male infertility idiopathic or NOS	Rheumatoid arthritis associated interstitial lung disease
Allergic rhinitis	Down's Syndrome in middle age	Malignancies	Rheumatoid spondylitis
Allergic skin reactions	Drug sensitivity	Malignant Lymphoma	Sarcoidosis
Allergy and asthma	Drug-Induced hepatitis	Malignant histiocytosis	Sarcomas
Allograft rejection	Drug-induced interstitial lung disease	Malignant melanoma	Schmidt's syndrome
Alopecia	Drug-induced movement disorders induced by drugs which block CNS	Memory Disorder	Scleroderma

Table 7. Diseases with Potential for β-NGF			
	dopamine receptors		
Alopecia areata	Duodenal ulcers	Meningitis	Sciatic neuropathy
Alpha-1-antitrypsin deficiency	Dysmenorrhoea	Meningococcemia	Senile Dementia of Lewy body type
Alzheimer's disease	Dyspepsia	Mental disorders (e.g., depression and schizophrenia)	Senile chorea
Amyotrophic lateral sclerosis	Eczema	Metabolic and idiopathic diseases	Sepsis syndrome
Anemia	Encephalomyelitis	Metabolic/idiopathic	Septic arthritis
Angina pectoris	Endocarditis	Microscopic vasculitis of the kidneys	Septic shock
Ankylosing spondylitis associated lung disease	Endocrinopathy	Migraine	Seronegative arthopathy
Anterior hom cell degeneration	Enteropathic synovitis	Mitochondrial multi-system disorder	Shock
Anti cd3 therapy	Epilepsy	Mixed connective tissue disease	Sickle cell anemia
Anti-receptor hypersensitivity reactions	Epiglottitis	Mixed connective tissue disease associated lung disease	Sjögren's disease associated lung disease
Antibody mediated cytotoxicity	Epithelial tissue damage or dysfunction	Mixed-vascular or non-vacular syndromes	Sjögren's syndrome
Antiphospholipid syndrome	Epstein-barr virus infection	Monoclonal gammopathy	Skin allograft rejection
Aordic and peripheral aneuryisms	Erythromelalgia	Multiple myeloma	Skin changes syndrome
Aortic dissection	Extrapyramidal and cerebellar disorders	Multiple sclerosis (all subtypes)	Skin complaints with inflammatory components
Arterial hypertension	Extramammary Paget's disease	Multiple systems degenerations (Mencel Dejerine-Thomas Shi-Drager and Machado-Joseph)	Small bowel transplant rejection
Arteriosclerosis	Familial hematophagocytic lymphohistiocytosis	Myalgic encephalitis/Royal Free Disease	Solid and liquid tumor pathologies
Arteriovenous fistula	Female infertility	Myasthenia gravis	Solid tumors
Arthritis	Fetal thymus implant rejection	Mycobacterium avium intracellulare	Specific arrythmias

Table 7. Diseases with Potential for β-NGF			
Arthropathy	Fibromyalgia	Mycobacterium tuberculosis	Sperm autoimmunity
Asthenia	Fibrosis	Myelodyplastic syndrome	Spinal ataxia
Asthma	Fibrotic lung disease	Myocardial infarction	Spinocerebellar degenerations
Ataxia	Friedreich's ataxia	Myocardial ischemic disorders	Spondyloarthopathy
Atheromatous disease/ arteriosclerosis	Functional peripheral arterial disorders	Myositis	Sporadic
Atherosclerosis	Fungal sepsis	β-NGF-related pain and hyperalgesia	Still's disease
Atopic allergy	Gas gangrene	Nasopharyngeal carcinoma	Streptococcal myositis
Atopic dermatitis	Gastric ulcer	Neonatal chronic lung disease	Stroke
Atrial fibrillation (sustained or paroxysmal)	Gastroesophageal reflux	Nephritis	Structural lesions of the cerebellum
Atrial flutter	Gastrointestinal or vascular regions	Nephrosis	Subacute sclerosing panencephalitis
Atrioventricular block	General gastrointestinal disorders	Nephrotic syndrome	Sunburn
Atrophic autoimmune hypothyroidism	General headache	Neuritis	Surgical pain
Autoimmune bullous disease	General inflammation	Neurodegenerative diseases	Sympathetic ophthalmia
Autoimmune diseases	Genitourinary	Neurogenic I muscular atrophies	Sympathetically maintained pain
Autoimmune haemolytic anemia	Giant cell arteritis	Neurological diseases	Syncope
Autoimmune hepatitis	Glomerular nephritis	Neuropathic pain	Syphilis of the cardiovascular system
Autoimmune mediated hypoglycemia	Goitrous autoimmune hypothyroidism (Hashimoto's disease)	Neuropathic pain and associated hyperalgesia and allodynia	Systemic anaphalaxis
Autoimmune neutropenia	Goodpasture's syndrome	Neuropathic pain and associated hyperalgesia or allodynia	Systemic inflammatory response syndrome
Autoimmune thrombocytopenia	Gouty arthritis	Neutropenic fever	Systemic lupus erythematosus

Table 7. Diseases with Potential for β-NGF			
Autoimmune thyroid disease	Graft rejection of any organ or tissue	Non-alcoholic Steatohepatitis	Systemic lupus erythematosus associated lung disease
B cell lymphoma	Glaucoma	Non-hodgkins lymphoma	Systemic onset juvenile rheumatoid arthritis
Bone graft rejection	Gram negative sepsis	Obstetric and gynecologic diseases	Systemic sclerosis
Bone marrow transplant (BMT) rejection	Gram positive sepsis	Occlusion of the abdominal aorta and its branches	Systemic sclerosis associated interstitial lung disease
Bronchial disorders	Granulomas due to intracellular organisms	Occulsive arterial disorders	T-cell or FAB ALL
Bronchiolitis obliterans	Grave's disease	Okt3 therapy	Takayasu's disease/arteritis
Bundle branch block	Group B streptococci (GBS) infection	Opthalmological diseases	Telangiectasia
Burkitt's lymphoma	HIV	Orchitis/epidydimitis	Tension headache
Burns	HIV neuropathy	Orchitis/vasectomy reversal procedures	Th2 Type and Th1 Type mediated diseases
Cachexia	Haemosiderosis associated lung disease	Organ transplant rejection	Thalamic pain syndrome
Cancer	Hairy cell leukemia	Organomegaly	Thromboangitis obliterans
Cardiac arrhythmias	Hallerrorden-Spatz disease	Osteoarthritis	Thrombocytopenia
Cardiac stun syndrome	Hashimoto's thyroiditis	Osteoarthrosis	Thyroiditis
Cardiac tumors	Hay fever	Osteoporosis	Toxic shock syndrome
Cardiomyopathy	Heart failure	Diseases of airway inflammation	Toxicity
Cardiopulmonary bypass inflammation response	Heart transplant rejection	Ovarian failure	Toxins
Cardiovascular affections	Hemachromatosis	POEMS syndrome (polyneuropathy, organomegaly, endocrinopathy, monoclonal gammopathy, and skin changes syndrome)	Toxins and chemotherapy

Table 7. Diseases with Potential for β-NGF			
Carditis	Hematopoietic malignancies (leukemia and lymphoma) Abetalipoprotemia	Pain	Transplant rejection diseases
Cartilage transplant rejection	Hemodialysis	Pain from amputation or abscess	Transplants
Causalgia	Hemolytic anemia	Pain from trauma	Trauma/hemorrhage
Cerebellar cortical degenerations	Hemolytic uremic syndrome/thrombolytic thrombocytopenic purpura, hemorrhage	Pancreas transplant rejection	Trigeminal neuralgia
Cerebellar disorders	Henoch-Schoenlein purpurea	Pancreatic carcinoma	Type B insulin resistance with acanthosis nigricans
Chaotic or multifocal atrial tachycardia	Hepatitis A	Pancreatitis	Type III hypersensitivity reactions
Chemotherapy	Hepatitis B	Paraneoplastic syndrome/hypercalcemia of malignancy	Type IV hypersensitivity
Cerebral Infarction	Hepatitis C	Parasitic diseases	Type-1 autoimmune hepatitis (classical autoimmune or lupoid hepatitis)
Chlamydia	Herpes simplex	Parathyroid transplant rejection	Type-2 autoimmune hepatitis (anti-LKM antibody hepatitis)
Choleosatatis	His bundle arrythmias	Parkinson's disease	Ulcerative colitic arthropathy
Chromic myelocytic leukemia (CML)	Hodgkin's disease	Pelvic inflammatory disease	Ulcerative colitis
Chronic active hepatitis	Huntington's chorea	Pemphigoid	Unstable angina
Chronic alcoholism	Hyperkinetic movement disorders	Pemphigus foliaceus	Uremia
Chronic eosinophilic pneumonia	Hypersensitity reactions	Pemphigus vulgaris	Urosepsis
Chronic fatigue syndrome	Hypersensitivity pneumonitis	Perennial rhinitis	Urticaria
Chronic immune disease associated with organ transplantation	Hypertension	Pericardial disease	Uveitis

Table 7. Diseases with Potential for β-NGF			
Chronic inflammatory conditions	Hyperthyroidism	Peripheral atherlosclerotic disease	Valvular heart diseases
Chronic inflammatory pain or neuropathic pain	Hypokinetic movement disorders	Peripheral vascular disorders	Varicose veins
Chronic inflammatory pathologies	Hypoglycemia	Peritonitis	Vasculitic diffuse lung disease
Chronic liver diseases	Hypoparathyroidism	Pernicious anemia	Vasculitis
Chronic lymphocytic leukemia (CLL)	Hypothalamic-pituitary-adrenal axis evaluation	Phacogenic uveitis	Vasomotor or allergic rhinitis
Chronic mucocutaneous candidiasis	Iatrogenic intoxication conditions	Pneumocystis carinii pneumonia	Venous diseases
Chronic obstructive pulmonary disease	Idiopathic Addison's disease	Pneumonia	Venous thrombosis
Chronic obstructive pulmonary disease (COPD)	Idiopathic leucopenia	Polyglandular deficiency type I and polyglandular deficiency type II	Ventricular fibrillation
Chronic salicylate intoxication	Idiopathic pulmonary fibrosis	Post herpetic neuralgia	Viral and fungal infections
Chronic visceral pain	Idiopathic thrombocytopenia	Post perfusion syndrome	Visceralgia or irritable bowel syndrome
Cluster headache	Idiosyncratic liver disease	Post pump syndrome	Vital encephalitis/aseptic meningitis
Colitis	Post-surgical pain	Post-MI cardiotomy syndrome	Vital-associated hemaphagocytic syndrome
Collagen vascular diseases	Infantile spinal muscular atrophy	Post-inflammatory interstitial lung disease	Vitiligo
Colorectal carcinoma	Infectious diseases	Postinfectious interstitial lung disease	Vitiligo acute liver disease
Common varied immunodeficiency (common variable hypogammaglobulinemia)	Inflammation of the aorta	Preeclampsia	Wallerian Degeneration
Congenital diseases	Inflammatory bowel disease	Premature ovarian failure	Wegener's granulomatosis
Congestive heart failure	Inflammatory bowel disorders	Primary biliary cirrhosis	Wernicke-Korsakoff syndrome
Conjunctivitis	Inflammatory	Primary myxoedema	Wilson's disease

Table 7. Diseases with Potential for β-NGF			
	diseases		
Connective tissue disease associated interstitial lung disease	Inflammatory eye disorders	Primary pulmonary hypertension	Wounds
Contact dermatitis	Inflammatory or unstable bladder disorders	Primary sclerosing cholangitis	Xenograft rejection of any organ or tissue
Coombs positive hemolytic anemia	Inflammatory pain	Primary sclerosing hepatitis	Yersinia and salmonella associated arthropathy
Cor pulmonale	Inflammatory pain and associated hyperalgesia and allodynia	Primary vasculitis	
Coronary artery disease	Influenza A	Progressive supranucleo Palsy	
Creutzfeldt-Jakob disease	Insulin dependent diabetes mellitus		

What is claimed is:

1. An aptamer comprised of the sequence BAZGRGGRSZWGGGGZZWADCCGZZRZG (SEQ ID NO: 45) or a fragment thereof,
wherein
B is selected from a C, G or Z;
R is independently selected from an A or G;
S is selected from a C or G;
W is independently selected from a Z or T;
D is selected from an A, G or Z; and
Z is independently selected from a modified pyrimidine, and wherein non-specific nucleotide insertions may be included.

2. The aptamer of claim 1, wherein the aptamer binds to β-NGF.

3. The aptamer of claim 2, wherein the aptamer inhibits the function of β-NGF.

4. The aptamer of claim 2, wherein said aptamer has the ability to modulate the binding of β-NGF to its one or more of its cellular receptors.

5. The aptamer of claim 4 wherein said cellular receptor is selected from p75 or TrkA.

6. The aptamer of claim 2, wherein the aptamer comprises a K_d for β-NGF of 30 nM or less.

7. The aptamer of claim 1, wherein said modified pyrimidine is a C-5 modified pyrimidine.

8. The aptamer of claim 7, wherein the C-5 modified pyrimidine is selected from the group consisting of Figure 10.

9. The aptamer of claim 7, wherein the C-5 modified pyrimidine is selected from the group consisting of 5-(N-benzylcarboxyamide)-2'-deoxyuridine (BndU), 5-(N-isobutylcarboxyamide)-2'-deoxyuridine (iBudU), 5-(N-tryptaminocarboxyamide)-2'-deoxyuridine (TrpdU) and 5-(N-naphthylmethylcarboxyamide)-2'-deoxyuridine (NapdU).

10. The aptamer of claim 7, wherein the C-5 modified pyrimidine is 5-(N-benzylcarboxyamide)-2'-deoxyuridine (BndU).

11. The aptamer of claim 1, wherein said aptamer includes at least one nucleotide insertion (N) and has the following sequence:

BAZGRGGRS$\mathbf{N}_{(0-1)}$ZWGGGGG$\mathbf{N}_{(0-1)}$ZZWADCCGZZRZG (SEQ ID NO:154)

wherein N is independently selected from any naturally occurring or modified nucleotide.

12. An aptamer comprised of the sequence BAZGRGGRSZZGGGGZZZADCCGZZRZG (SEQ. ID. NO: 3), or a fragment thereof,
wherein
B is selected from a C, G or Z;
R is independently selected from an A or G;
S is selected from a C or G;
D is selected from an A, G or Z; and
Z is independently selected from a modified pyrimidine, and wherein non-specific nucleotide insertions may be included.

13. The aptamer of claim 12, wherein the aptamer binds to β-NGF.

14. The aptamer of claim 13, wherein the aptamer inhibits the function of β-NGF.

15. The aptamer of claim 13, wherein said aptamer has the ability to modulate the binding of β-NGF to its one or more of its cellular receptors.

16. The aptamer of claim 15 wherein said cellular receptor is selected from p75 or TrkA.

17. The aptamer of claim 13, wherein the aptamer comprises a K_d for β-NGF of 30 nM or less.

18. The aptamer of claim 12, wherein said modified pyrimidine is a C-5 modified pyrimidine.

19. The aptamer of claim 18, wherein the C-5 modified pyrimidine is selected from the group consisting of Figure 10.

20. The aptamer of claim 18, wherein the C-5 modified pyrimidine is selected from the group consisting of 5-(N-benzylcarboxyamide)-2'-deoxyuridine (BndU), 5-(N-isobutylcarboxyamide)-2'-deoxyuridine (iBudU), 5-(N-tryptaminocarboxyamide)-2'-deoxyuridine (TrpdU) and 5-(N-naphthylmethylcarboxyamide)-2'-deoxyuridine (NapdU).

21. The aptamer of claim 18, wherein the C-5 modified pyrimidine is 5-(N-benzylcarboxyamide)-2'-deoxyuridine (BndU).

22. The aptamer of claim 18, wherein said aptamer includes at least one nucleotide insertion (N) and has the following sequence:

BAZGRGGRS$\mathbf{N}_{(0-1)}$ZZGGGGG$\mathbf{N}_{(0-1)}$ZZZADCCGZZRZG (SEQ ID NO: 155)

wherein N is independently selected from any naturally occurring or modified nucleotide.

23. An aptamer that binds to β-NGF comprising a sequence selected from the group consisting of SEQ. ID. NOS: 1, 2, 9-44 and 149.

24. The aptamer of claim 23, wherein the aptamer inhibits the function of β-NGF.

25. The aptamer of claim 23, wherein said aptamer has the ability to modulate the binding of β-NGF to its one or more of its cellular receptors.

26. The aptamer of claim 25, wherein said cellular receptor is selected from p75 or TrkA.

27. The aptamer of claim 26, wherein the sequence is selected from the group consisting of at least about 90% identity, at least about 91% identity, at least about 92% identity, at least about 93% identity, at least about 94% identity, and at least about 95% identity.

28. A method for treating, preventing or ameliorating a disease mediated by β-NGF comprising administering to a subject in need thereof a therapeutically effective dose of an

aptamer that binds to β-NGF comprised of the sequence BAZGRGGRSZWGGGGZZWADCCGZZRZG (SEQ ID NO: 45) or a fragment thereof, wherein

B is selected from a C, G or Z;

R is independently selected from an A or G;

S is selected from a C or G;

W is independently selected from a Z or T;

D is selected from an A, G or Z; and

Z is independently selected from a modified pyrimidine, and wherein non-specific nucleotide insertions may be included.

29. The method of claim 28, wherein said modified pyrimidine is a C-5 modified pyrimidine.

30. The method of claim 29, wherein the C-5 modified pyrimidine is selected from the group consisting of Figure 10.

31. The method of claim 29, wherein the C-5 modified pyrimidine is selected from the group consisting of 5-(N-benzylcarboxyamide)-2'-deoxyuridine (BndU), 5-(N-isobutylcarboxyamide)-2'-deoxyuridine (iBudU), 5-(N-tryptaminocarboxyamide)-2'-deoxyuridine (TrpdU) and 5-(N-naphthylmethylcarboxyamide)-2'-deoxyuridine (NapdU).

32. The method of claim 29, wherein the C-5 modified pyrimidine is 5-(N-benzylcarboxyamide)-2'-deoxyuridine (BndU).

33. The method of claim 28, wherein the β-NGF mediated disease or condition is one in which β-NGF activity may directly or indirectly lead to pruritus at some stage in the disease process.

34. The method of claim 33, wherein the disease or condition is dermatitis or eczema.

35. The method of claim 34, wherein the dermatitis is atopic dermatitis.

36. The method of claim 28, wherein the aptamer is selected from the group consisting of SEQ ID NOS: 1, 2, 9-44 and 149.

37. The method of claim 36, wherein the sequence is selected from the group consisting of at least about 90% identity, at least about 91% identity, at least about 92% identity, at least about 93% identity, at least about 94% identity, and at least about 95% identity.

38. The method of claim 28, wherein said administration is topical.

39. The method of claim 28, wherein the aptamer is administered at a dose in the range of about 1 μg to about 100 mg/kg body weight per day.

40. The method of claim 28, wherein the aptamer is administered in combination with another agent used in the treatment of pruritus.

41. The method of claim 28, wherein said aptamer includes at least one nucleotide insertion (N) and has the following sequence:

BAZGRGGRS$\mathbf{N}_{(0\text{-}1)}$ZWGGGG$\mathbf{N}_{(0\text{-}1)}$ZZWADCCGZZRZG (SEQ ID NO: 154)

wherein N is independently selected from any naturally occurring or modified nucleotide.

42. A method for treating, preventing or ameliorating a disease mediated by β-NGF comprising administering to a subject in need thereof a therapeutically effective dose of an aptamer that binds to β-NGF comprised of the sequence BAZGRGGRSZZGGGGZZZADCCGZZRZG (SEQ ID NO: 3), or a fragment thereof, wherein

B is selected from a C, G or Z;

R is independently selected from an A or G;

S is selected from a C or G;

D is selected from an A, G or Z; and

Z is independently selected from a modified pyrimidine, and wherein non-specific nucleotide insertions may be included.

43. The method of claim 42, wherein said modified pyrimidine is a C-5 modified pyrimidine.

44. The method of claim 43, wherein the C-5 modified pyrimidine is selected from the group consisting of Figure 10.

45. The method of claim 43, wherein the C-5 modified pyrimidine is selected from the group consisting of 5-(N-benzylcarboxyamide)-2'-deoxyuridine (BndU), 5-(N-isobutylcarboxyamide)-2'-deoxyuridine (iBudU), 5-(N-tryptaminocarboxyamide)-2'-deoxyuridine (TrpdU) and 5-(N-naphthylmethylcarboxyamide)-2'-deoxyuridine (NapdU).

46. The method of claim 43, wherein the C-5 modified pyrimidine is 5-(N-benzylcarboxyamide)-2'-deoxyuridine (BndU).

47. The method of claim 42, wherein the β-NGF mediated disease or condition is one in which β-NGF activity may directly or indirectly lead to pruritus at some stage in the disease process.

48. The method of claim 47, wherein the disease or condition is dermatitis or eczema.

49. The method of claim 48, wherein the dermatitis is atopic dermatitis.

50. The method of claim 42, wherein said administration is topical.

51. The method of claim 42, wherein the aptamer is administered at a dose in the range of about 1 μg to about 100 mg/kg body weight per day.

52. The method of claim 42, wherein the aptamer is administered in combination with another agent used in the treatment of pruritus.

53. The method of claim 42, wherein said aptamer includes at least one nucleotide insertion (N) and has the following sequence:

BAZGRGGRS$\mathbf{N}_{(0\text{-}1)}$ZZGGGGG$\mathbf{N}_{(0\text{-}1)}$ZZZADCCGZZRZG (SEQ ID NO: 155)

wherein N is independently selected from any naturally occurring or modified nucleotide.

54. A pharmaceutical composition comprising an aptamer to β-NGF comprised of the sequence BAZGRGGRSZWGGGGZZWADCCGZZRZG (SEQ. ID. NO: 45), or a pharmaceutically acceptable salt thereof or a fragment thereof:
wherein
B is selected from a C, G or Z;
R is independently selected from an A or G;
S is selected from a C or G;
W is independently selected from a Z or T;
D is selected from an A, G or Z; and
Z is independently selected from a modified pyrimidine, and wherein non-specific nucleotide insertions may be included and a pharmaceutically acceptable carrier or excipient.

55. The composition of claim 54, further comprising a diluent, antimicrobial agent, a binding agent, a filler, a wetting agent, a suspending agent, an emollient, and an emulsifying agent.

56. The composition of claim 55, wherein said formulation is a topical formulation selected from the group consisting of a cream, a lotion, an ointment, a gel, an impregnated dressing, an aerosol, a medicated powder, a medicated adhesive, and a foam.

57. A pharmaceutical composition comprising an aptamer to β-NGF comprised of the sequence BAZGRGGRSZZGGGGZZZADCCGZZRZG (SEQ ID NO: 3), or a pharmaceutically acceptable salt thereof or a fragment thereof:
wherein
B is selected from a C, G or Z;
R is independently selected from an A or G;
S is selected from a C or G;
D is selected from an A, G or Z; and
Z is independently selected from a modified pyrimidine, and wherein non-specific nucleotide insertions may be included and a pharmaceutically acceptable carrier or excipient.

58. The composition of claim 57, further comprising a diluent, antimicrobial agent, a binding agent, a filler, a wetting agent, a suspending agent, an emollient, and an emulsifying agent.

59. The composition of claim 58, wherein said formulation is a topical formulation selected from the group consisting of a cream, a lotion, an ointment, a gel, an impregnated dressing, an aerosol, a medicated powder, a medicated adhesive, and a foam.

60. The aptamer of claim1, wherein the aptamer is a dimer comprising two components, and wherein said two components are linked.

61. The aptamer of claim 19, wherein said two components are linked by a hydrocarbon linker.

62. An aptamer comprised of the sequence BAZGRGGRSZWGGGGZZWADCCGZZRZG (SEQ ID NO: 45) or a fragment thereof,
wherein
B is selected from any nucleotide other than A;
R is independently selected from an A or G;
S is selected from a C or G;
W is independently selected from a Z or T;
D is selected from an A, G or Z; and
Z is independently selected from a modified pyrimidine, and wherein non-specific nucleotide insertions may be included.

63. A pharmaceutical agent comprising an aptamer of any one of claims 1 to 27 as an active agent.

64. The pharmaceutical agent of claim 63 for treating, preventing or ameliorating a disease mediated by β-NGF comprising an aptamer.

65. The pharmaceutical agent of claim 64, wherein the β-NGF mediated disease or condition is one in which β-NGF activity may directly or indirectly lead to pruritus at some stage in the disease process.

66. The pharmaceutical agent of claim 63 or 65, wherein the disease or condition is dermatitis or eczema.

67. The pharmaceutical agent of claim 66, wherein the dermatitis is atopic dermatitis.

68. Use of an aptamer of any one of claims 1 to 27 for the production of a pharmaceutical agent.

69. The use of claims 68, wherein the pharmaceutical agent is an agent for treating, preventing or ameliorating a disease mediated by β-NGF comprising an aptamer.

70. The use of claim 69, wherein the β-NGF mediated disease or condition is one in which β-NGF activity may directly or indirectly lead to pruritus at some stage in the disease process.

71. The use of claim 68 or 70, wherein the disease or condition is dermatitis or eczema.

72. The use of claim 71, wherein the dermatitis is atopic dermatitis.

73. The aptamer of claims 1 to 27 for use in treating, preventing or ameliorating a disease mediated by β-NGF comprising an aptamer.

74. The aptamer of claim 73, wherein the β-NGF mediated disease or condition is one in which β-NGF activity may directly or indirectly lead to pruritus at some stage in the disease process.

75. The aptamer of claim 74, wherein the disease or condition is dermatitis or eczema.

76. The aptamer of claim 75, wherein the dermatitis is atopic dermatitis.

77. The composition of any one of claims 54 to 59 for treating, preventing or ameliorating a disease mediated by β-NGF.

78. The composition of claim 77, wherein the β-NGF mediated disease or condition is one in which β-NGF activity may directly or indirectly lead to pruritus at some stage in the disease process.

79. The composition of claim 78, wherein the disease or condition is dermatitis or eczema.

80. The composition of claim 79, wherein the dermatitis is atopic dermatitis.

81. The composition of any of claims 54 to 59 for treating, preventing or ameliorating a disease or condition is dermatitis or eczema.

82. The composition of claim 81, wherein the dermatitis is atopic dermatitis.

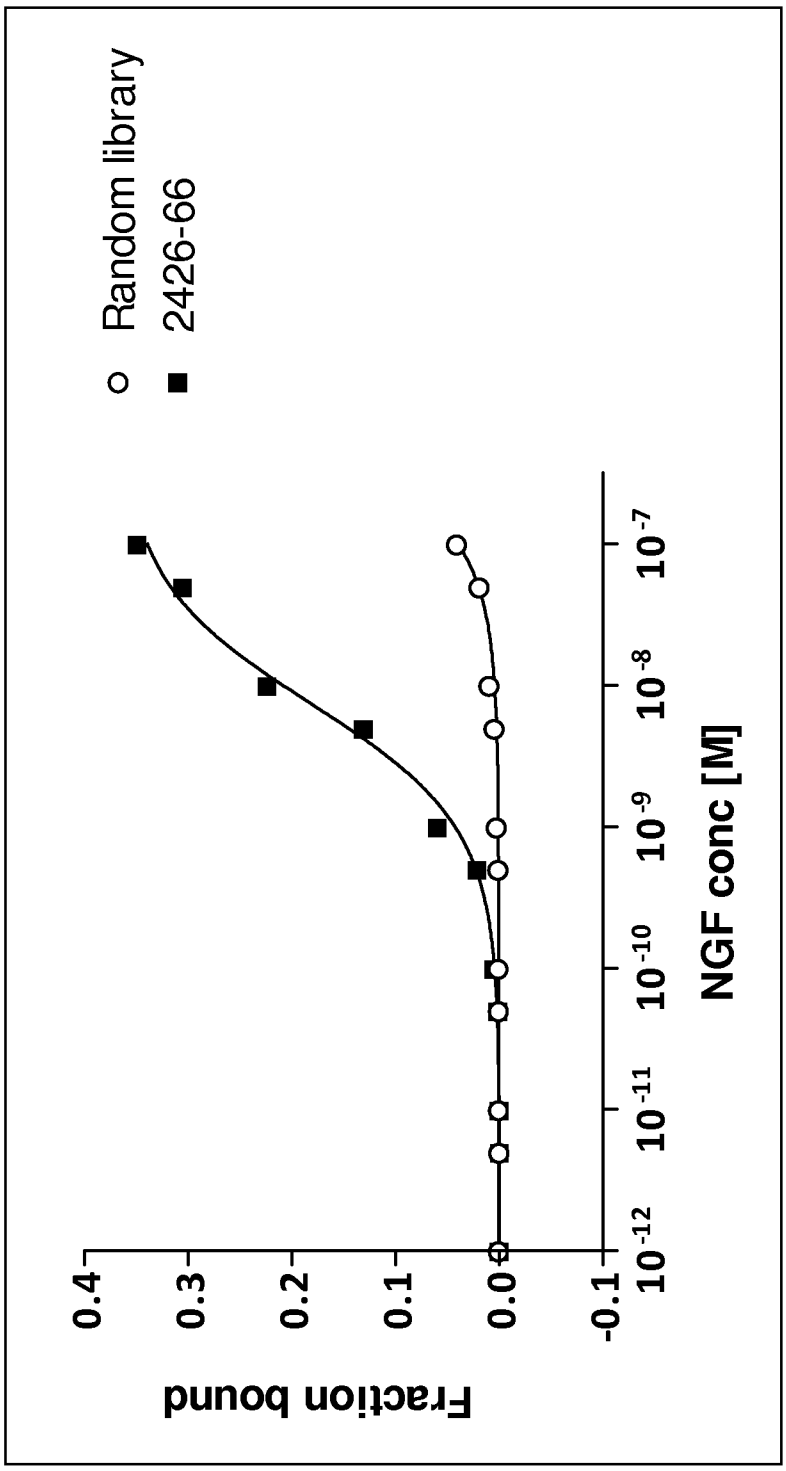

Random library
2426-66
Fraction bound
0.4
0.3
0.2
0.1
0.0
-0.1
10^{-12}
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
NGF conc [M]

FIG. 1

FIG. 2A

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Row (A)																												
A	0	92	0	0	61	0	0	75	2	1	0	0	0	1	0	0	0	1	96	13	0	0	3	0	0	20	0	1
Z	23	0	99	1	0	2	3	2	0	97	97	2	0	0	0	99	99	87	0	13	0	0	1	94	93	0	89	7
G	17	7	0	98	38	97	96	20	10	0	2	97	99	98	96	0	0	7	2	71	0	1	89	0	0	71	0	62
C	57	0	0	0	0	0	0	0	86	0	0	0	0	0	0	0	0	0	0	0	97	93	0	0	0	0	0	6
T											●							●										

Consensus	B	A	Z	G	R	G	G	R	S	Z	W	G	G	G	G	Z	Z	W	A	D	C	C	G	Z	Z	R	Z	G

indicates the frequency at which A is observed in the 2426-66 aptamer family at each of the 28 conserved positions
Row (Z) indicates the frequency at which Z is observed in the 2426-66 aptamer family at each of the 28 conserved positions
Row (G) indicates the frequency at which G is observed in the 2426-66 aptamer family at each of the 28 conserved positions
Row (C) indicates the frequency at which C is observed in the 2426-66 aptamer family at each of the 28 conserved positions
Row (T) indicates the only two conserved Z positions that can be replaced with T with no loss of β-NGF binding activity (●)

Row (Consensus) is the consensus sequence for the aptamer with the BzdU substitution, where:

Z = modified U
B = any nucleotide other than A
R = A or G
S = C or G
W = Z or T
D = any nucleotide other than C

FIG. 2B

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
A	0	92	0	0	0	61	0	0	0	75	2	0	1	0	0	0	1	0	0	0	0	1	96	13	0	0	0	3	0	0	0	20	0	1
Z	23	0	0	99	1	0	1	2	3	2	0	1	97	97	2	0	0	0	0	99	99	87	0	13	0	0	0	1	94	93	0	0	89	7
G	17	7	1	0	98	38	0	97	96	20	10	6	0	2	97	99	98	96	3	0	0	7	2	71	0	0	1	89	0	0	1	71	0	62
C	57	0	0	0	0	0	0	0	0	0	86	0	0	0	0	0	0	0	0	0	0	0	0	0	0	97	93	0	0	0	0	0	0	6
-	0	0	98	0	0	0	97	0	0	0	0	91	0	0	0	0	0	3	95	0	0	3	0	0	97	1	2	4	4	6	98	6	8	20

Consensus	B	A	-	Z	G	R	-	G	G	R	S	-	Z	Z	G	G	G	G	-	Z	Z	Z	A	D	-	C	C	G	Z	Z	-	R	Z	G

Row (A) indicates the frequency at which A is observed in the 2426-66 aptamer family at each of the 34 conserved positions
Row (Z) indicates the frequency at which Z is observed in the 2426-66 aptamer family at each of the 34 conserved positions
Row (G) indicates the frequency at which G is observed in the 2426-66 aptamer family at each of the 34 conserved positions
Row (C) indicates the frequency at which C is observed in the 2426-66 aptamer family at each of the 34 conserved positions
Row (-) indicates the frequency at which a deletion is observed in the 2426-66 aptamer family at each of the 34 conserved positions

Row (Consensus) is the consensus sequence for the aptamer with the BzdU substitution, where:

Z = modified U
B = C, G or Z
R = A or G
S = C or G
D = A, G or Z

FIG. 3

Dimerization Strategy #1

Head-to-tail NGF Dimers

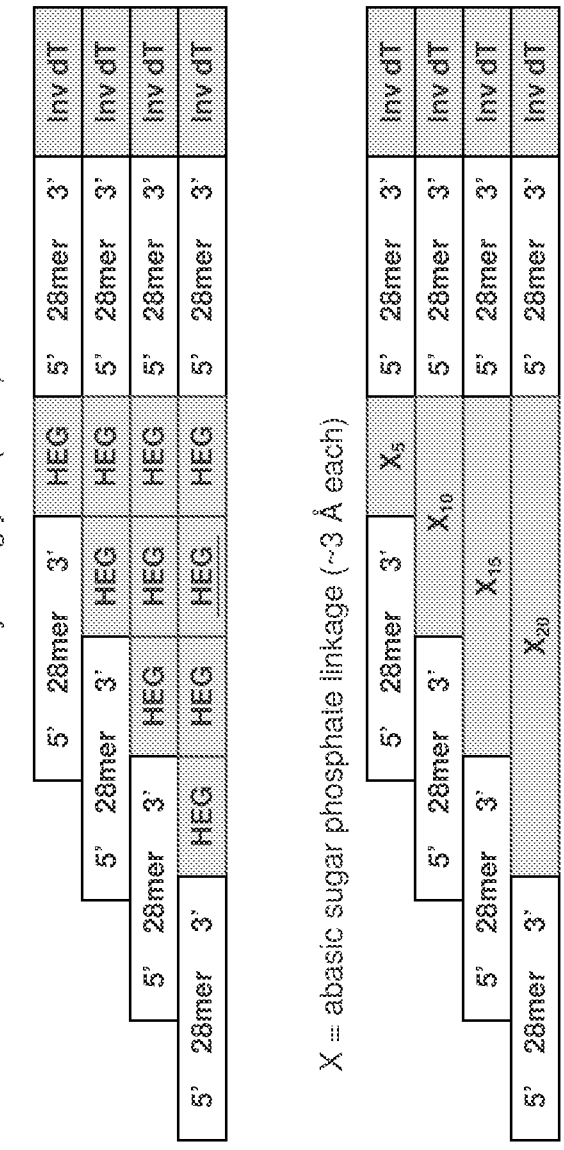
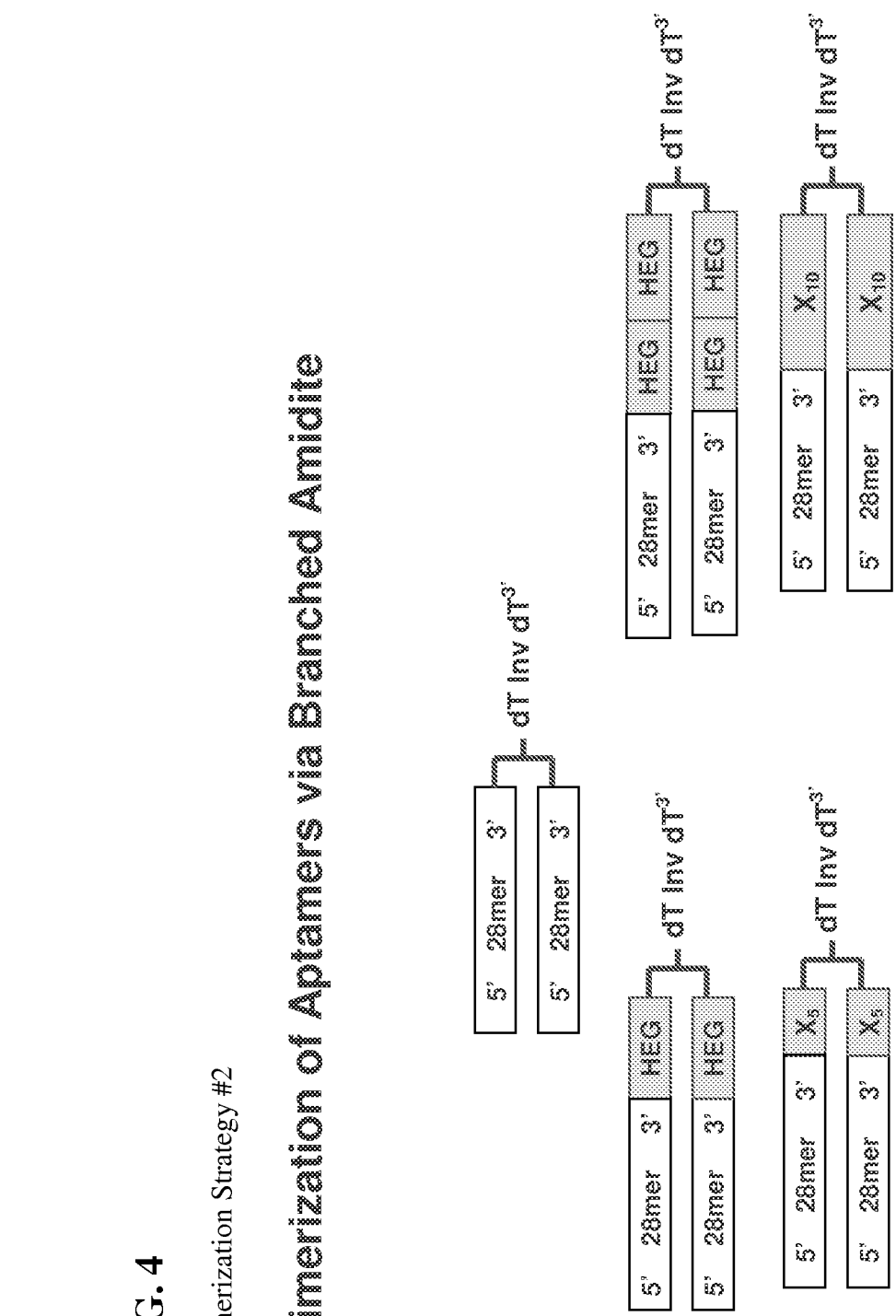


FIG. 4

Dimerization Strategy #2



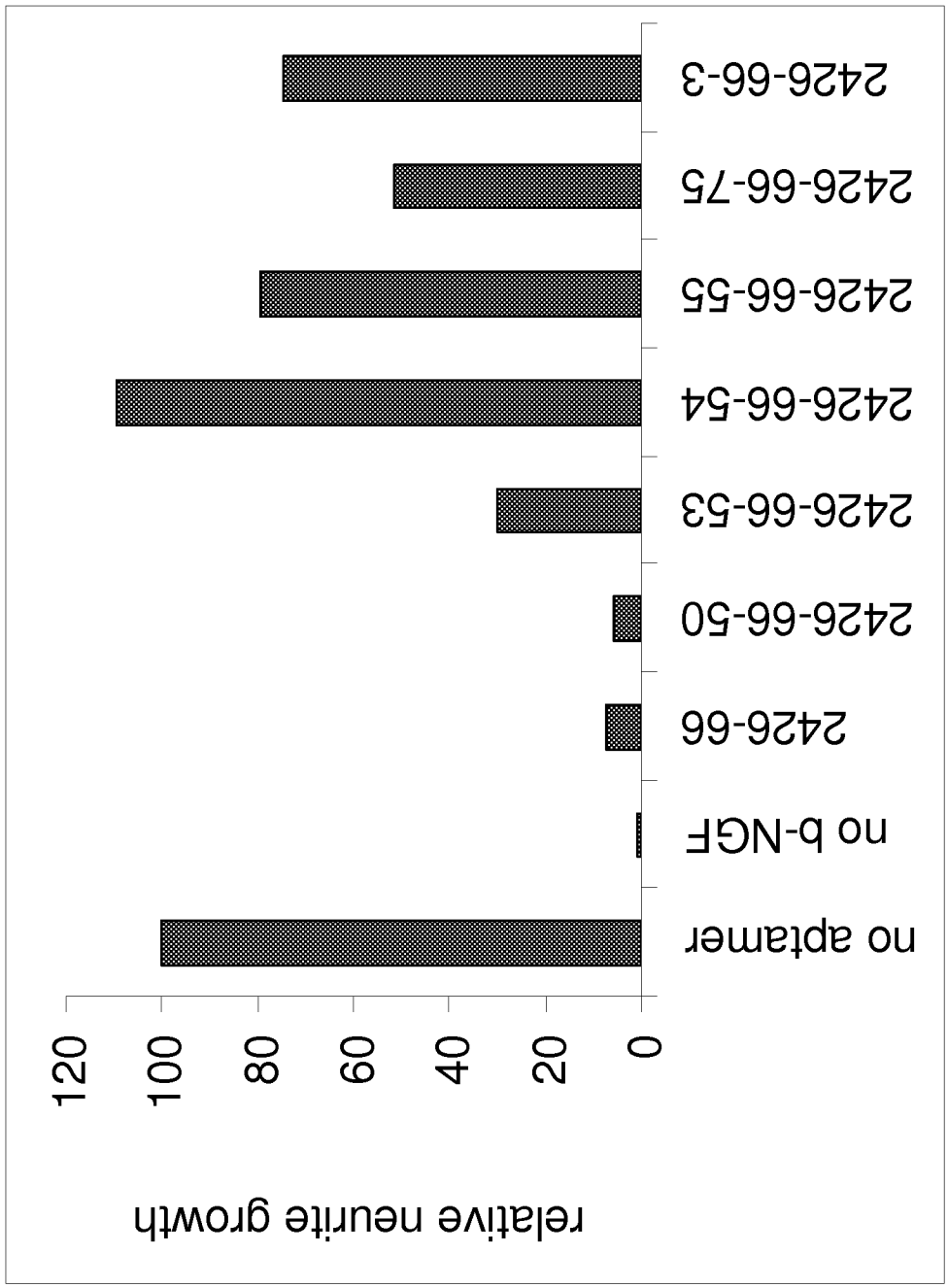

relative neurite growth
120
100
80
60
40
20
0
no aptamer
no b-NGF
2426-66
2426-66-50
2426-66-53
2426-66-54
2426-66-55
2426-66-75
2426-66-3

FIG. 5

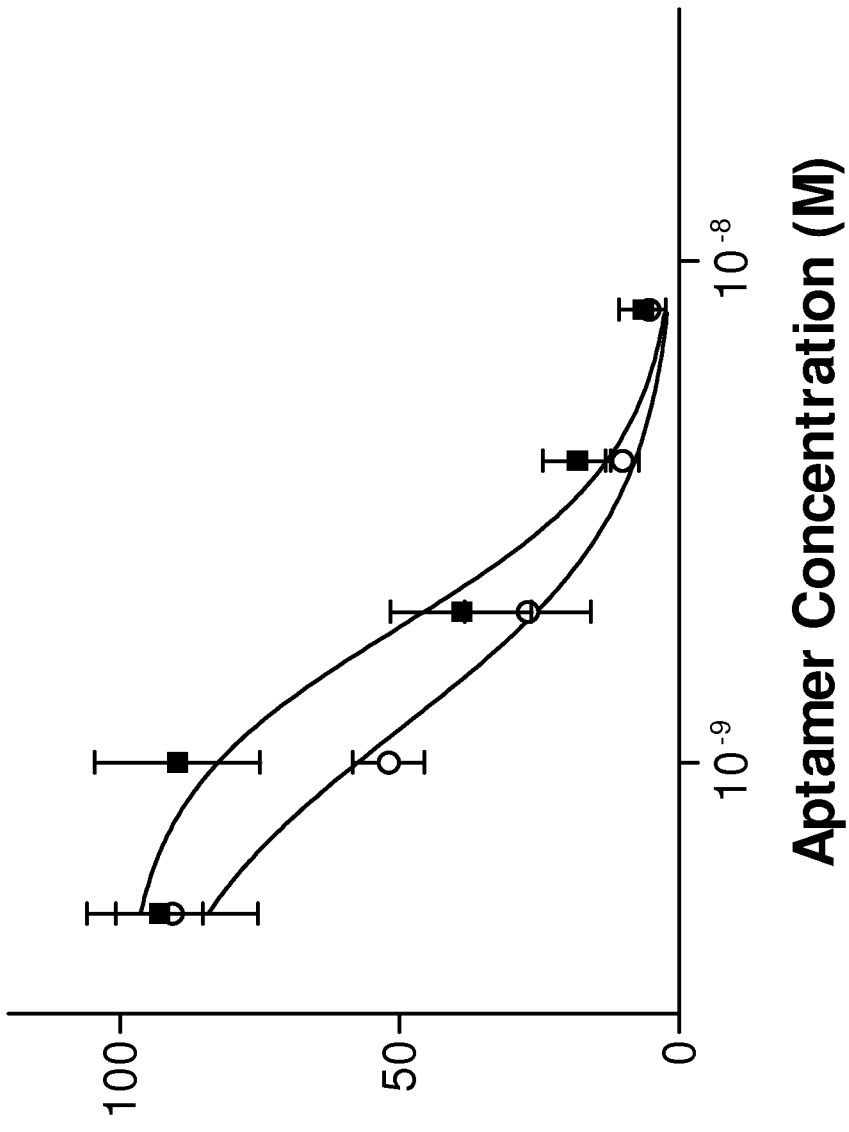

relative neurite growth
100
50
0
10^{-9}
10^{-8}
Aptamer Concentration (M)

FIG. 6

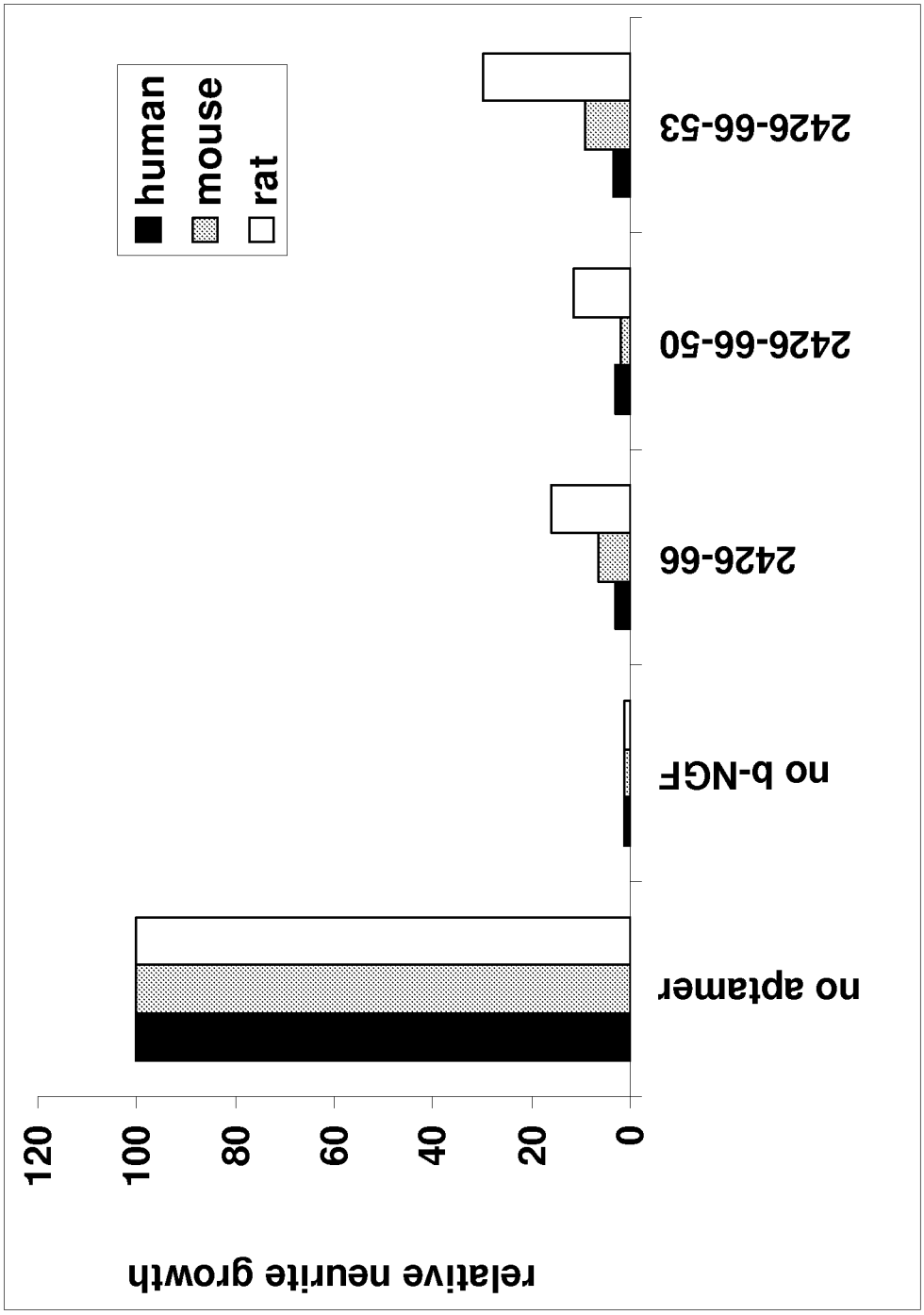

relative neurite growth
120
100
80
60
40
20
0
human
mouse
rat
no aptamer
no b-NGF
2426-66
2426-66-50
2426-66-53

FIG. 7

FIG. 8

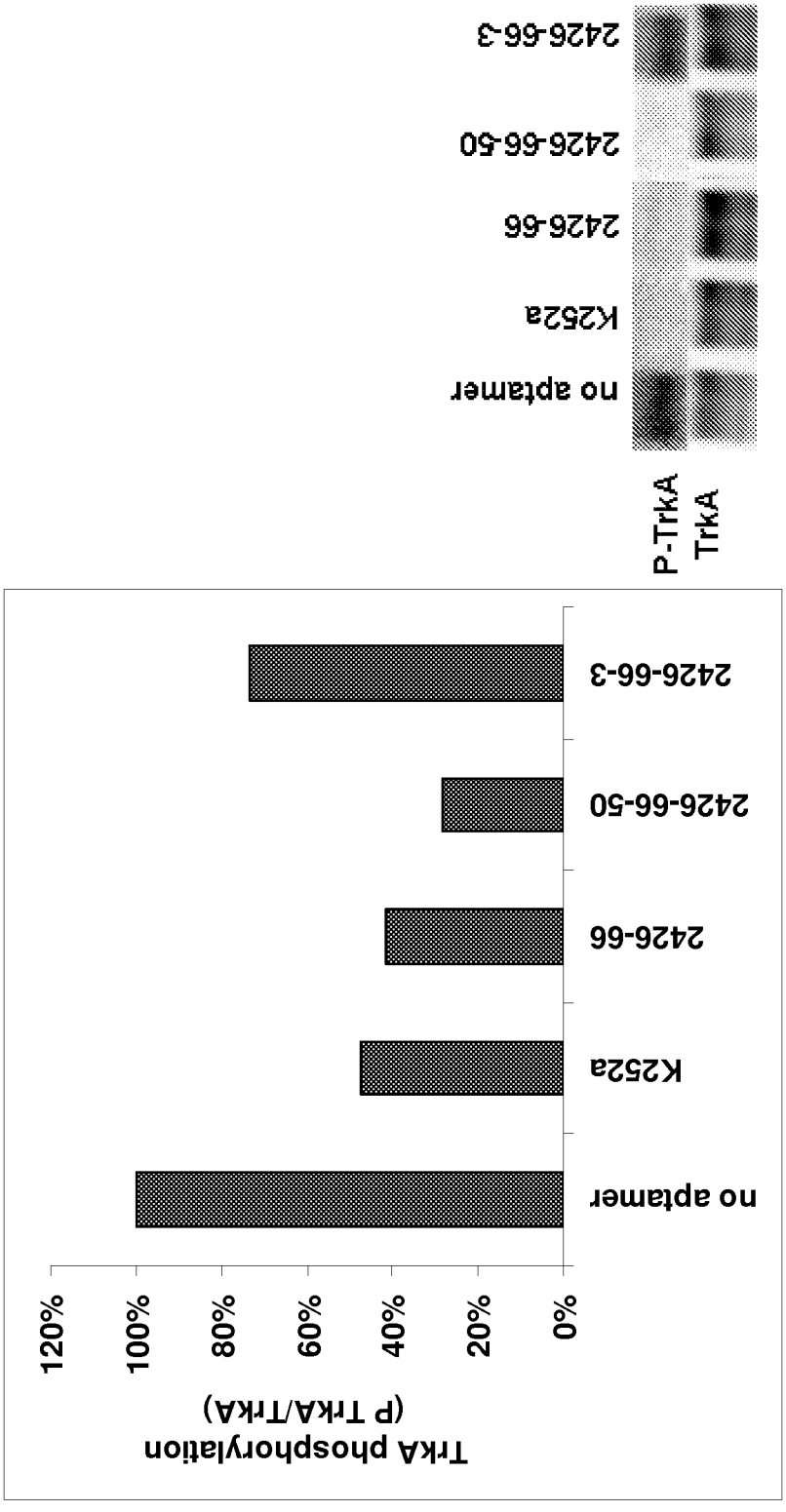

TrkA phosphorylation
(P TrkA/TrkA)
120%
100%
80%
60%
40%
20%
0%
no aptamer
K252a
2426-66
2426-66-50
2426-66-3
P-TrkA
TrkA
no aptamer
K252a
2426-66
2426-66-50
2426-66-3

FIG. 9

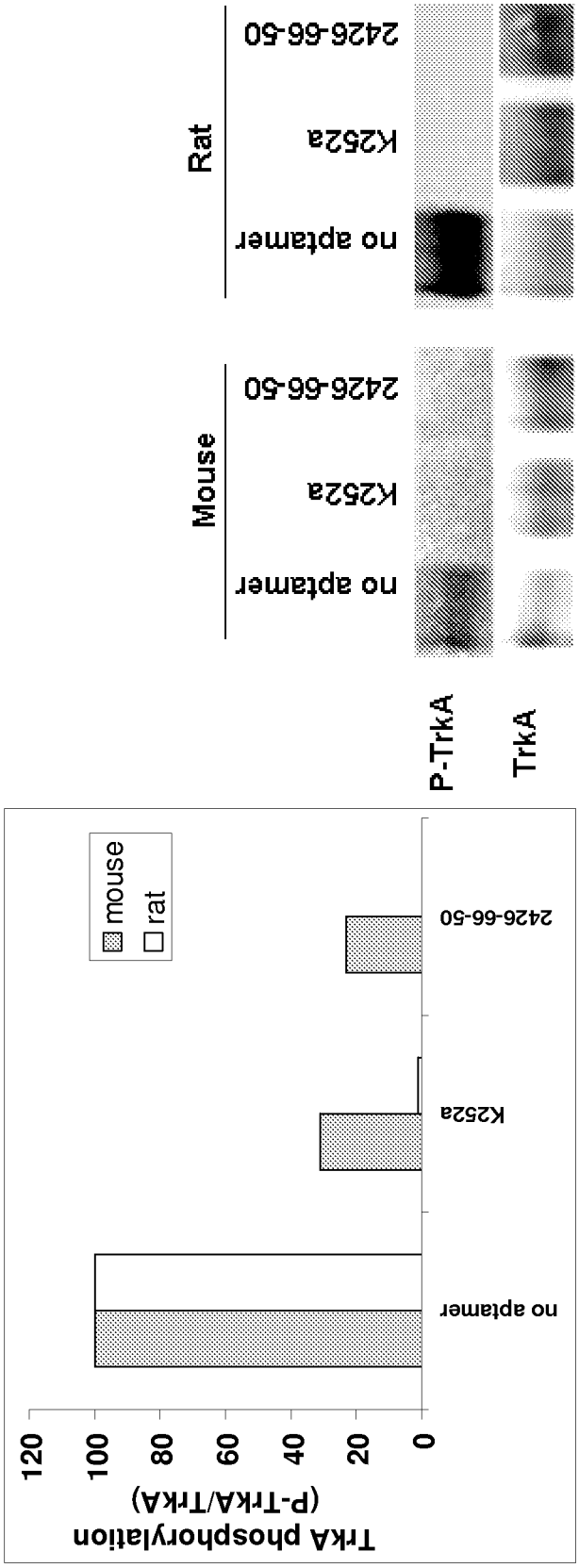
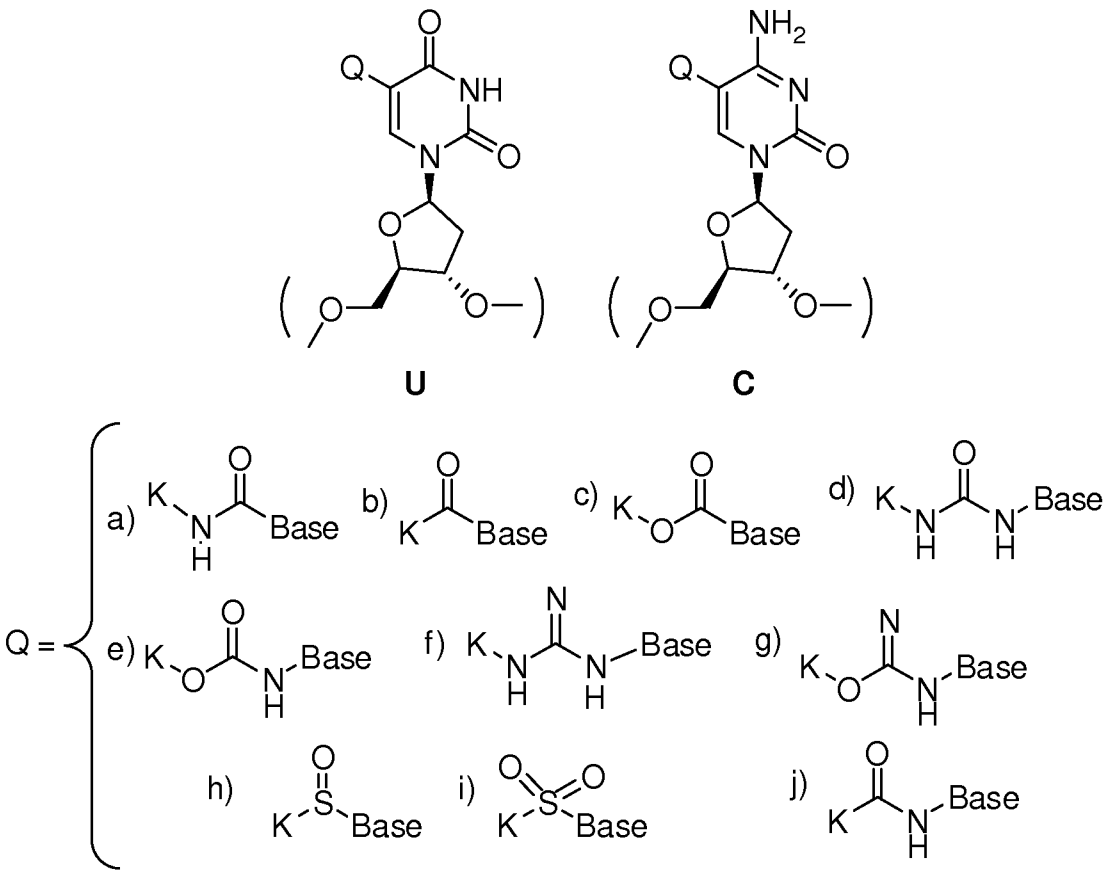

TrkA phosphorylation
(P-TrkA/TrkA)
120
100
80
60
40
20
0
mouse
rat
no aptamer
K252a
2426-66-50
P-TrkA
TrkA
Mouse
no aptamer
K252a
2426-66-50
Rat
no aptamer
K252a
2426-66-50

FIG. 10



Base = Uridine (U) or Cytidine(C) (attachment is to the 5-position)
K = R' group plus $(CH_2)_n$ connecting group, where n = 0-3

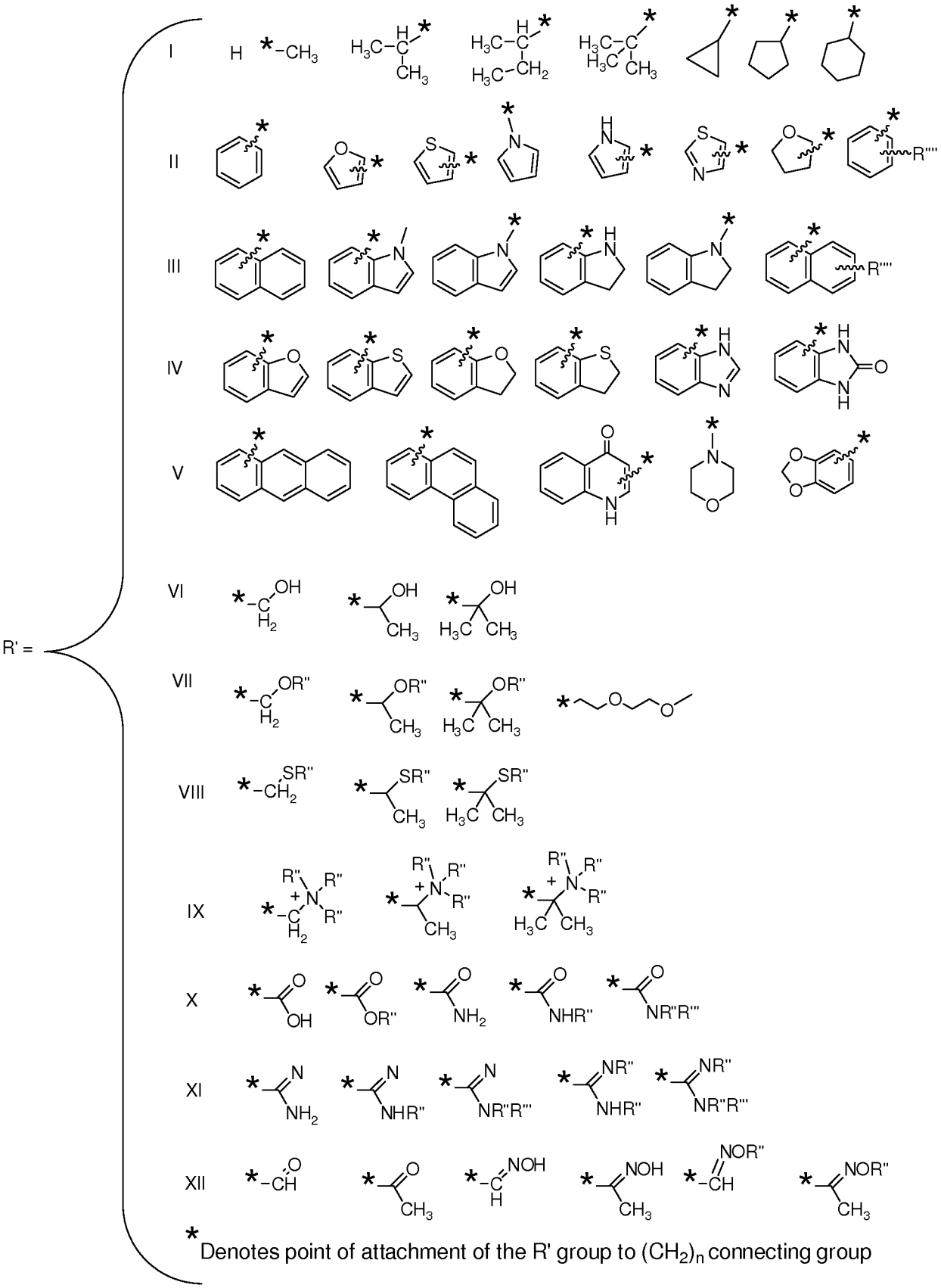


FIG. 10 continued

FIG. 10 continued

wherein

R"" is selected from the group consisting of a branched or linear lower alkyl (C1-C20); halogen (F, Cl, Br, I); nitrile (CN); boronic acid (BO_2H_2); carboxylic acid (COOH); carboxylic acid ester (COOR"); primary amide ($CONH_2$); secondary amide (CONHR"); tertiary amide (CONR"R'"); sulfonamide (SO_2NH_2); N-alkylsulfonamide (SONHR");

wherein

R", R'" are independently selected from a group consisting of a branched or linear lower alkyl (C1-C2)); phenyl (C6H5); an R"" substituted phenyl ring (R""C6H4); wherein R"" is defined above; a carboxylic acid (COOH); a carboxylic acid ester (COOR""); wherein R"" is a branched or linear lower alkyl (C1-C20); and cycloalkyl; wherein R" = R'" = (CH2)n;

wherein n =2-10.

FIG. 11

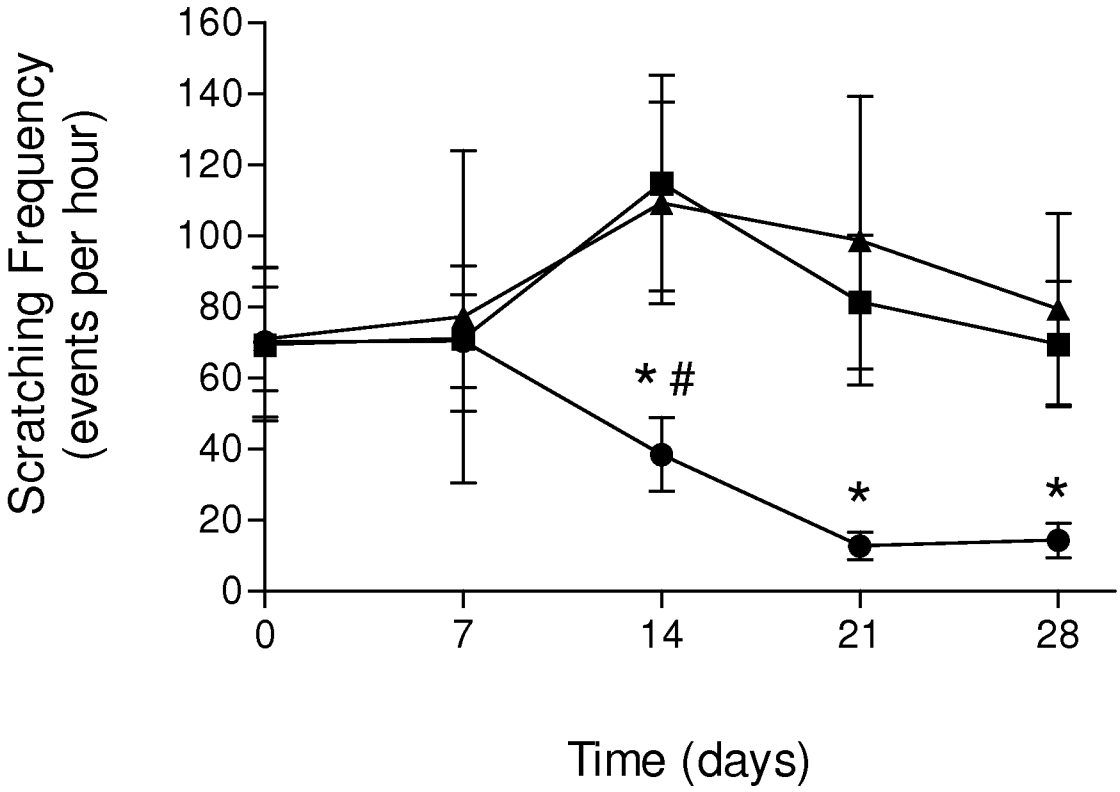
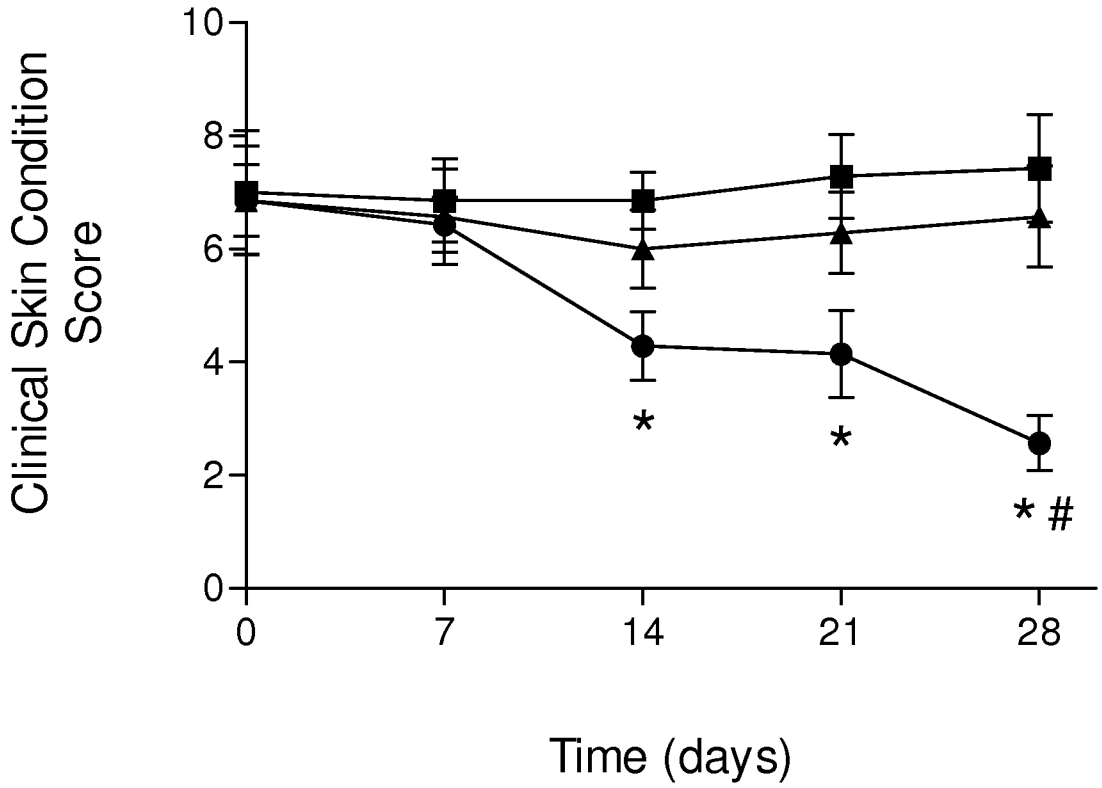

Scratching Frequency
(events per hour)
160
140
120
100
80
60
40
20
0
0
7
14
21
28
* #
*
*
Time (days)

FIG. 12



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 11/32017

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12P 19/34; C07H 21/04 (2011.01)
USPC - 435/91.1, 536/23.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - C12P 19/34; C07H 21/04 (2011.01)
USPC - 435/91.1, 536/23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 435/6, 91.1, 536/23.1
(Text Search)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST (PGPB, USPT, USOC, EPAB JPAB); Google Scholar; PubMed and GenCore.
Search Terms: aptamer, nerve growth factor, NGF, inhibi$, beta, modif$, C-5, pyrimidine, receptor, growth factor, bind, bind$, b-NGF, TrkA, p75, pruritus, atopic dermatitis, dermatitis, dimer, link$, linker, hydrocarbon, multivalent, alkan$, alken$, alkyn$, alky, carbon.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	US 2003/0054360 A1 (GOLD et al.) 20 March 2003 (20.03.2003) para [0004], [0024]-[0026], [0029], [0044], [0048], [0056], [0062], [0383].	1-2, 6-7, 11-13, 17-18, 22-23 and 62 ---------- 3-5, 8-10, 14-16, 19-21, 24-26, 28-36, 38-61, 63/(1-26)-65/(1-26), 68/(1-26)-70/(1-26), 73/(1-26)-76/(1-26) and 77-82
Y	US 2004/0120891 A1 (HILL et al.) 24 June 2004 (24.06.2004) para [0017], [0021], [0092], [0099].	3-5, 14-16, 24-26, 28-36, 38-59, 63/(1-26)-65/(1-26),68/(1-26)-70/(1-26), 73/(1-26)-76/(1-26) and 77-82
Y	US 2009/0004667 A1 (ZICHI et al.) 01 January 2009 (01.01.2009) fig 14; [0010], [0036], [0052], [0055].	8-10, 19-21, 30-32, 44-46 63/(8-10, 19-21)-65/(8-10, 19-21), 68/(8-10, 19-21)-70/(8-10, 19-21), 73/(8-10, 19-21)-76/(8-10, 19-21)

☒ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
08 June 2011 (08.06.2011)	17 JUN 2011
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 11/32017

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2007/0286915 A1 (TONOGAITO et al.) 13 December 2007 (13.12.2007) table 4; para [0009], [0015], [0023], [0036], [0037], [0040].	28-36, 38-59, 63/(1-26)-65/(1-26), 68/(1-26)-70/(1-26), 73/(1-26)-76/(1-26) and 77-82
Y	US 2008/0207523 A1 (FRIEBE et al.) 28 August 2008 (28.08.2008) table 7; para [0012], [0016], [0028], [0155], [0156].	60-61

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 11/32017

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 27 and 37
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claims 27 and 37 does not specify the SEQ ID Nos of the sequences to which the aptamers of claims 26 and 36 are compared to, and since a total of 169 sequences were submitted, no meaningful international search can be carried out without the specific SEQ ID NOs.

3. ☒ Claims Nos.: 66-67 and 71-72
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.