

TITLE: New TLR-4 antagonist aptamers

FIELD OF THE INVENTION

- 5 The present invention relates to the fields of oligonucleotides and therapeutic aptamers for human health, particularly useful in the treatment of TLR4-related diseases.

BACKGROUND ART

- 10 Toll-like receptors (TLRs) are a family of receptors that participate in innate immunity, stimulating a wide variety of inflammatory responses. TLR-4 activation increases the expression and nuclear translocation of nuclear transcription factor *kappa*-B (NF- κ B), leading to the release of proinflammatory cytokines like TNF- α , interleukin-1 β (IL-1 β) or interleukin-6 (IL-6), as well as chemokines and immune cells recruitment.

- 15 The possibility of down-regulating immune responses with specific TLR antagonists, inhibiting specific intracellular proteins involved in these signaling pathways, has raised great interest for the treatment of diseases with an inflammatory component. Aptamers offer several advantages over antibodies that make them ideal for future therapeutic applications, such as their high specificity and affinity, lack of immunogenicity and their ease to enter biological compartments given their small size.

- Patent document WO2015/197706A1 describes TLRApt#4F-T (SEQ ID NO: 2), a single-stranded DNA aptamer selected using the systemic evolution of ligands by exponential enrichment (SELEX) technology, which is an antagonist of TLR-4 with an immunomodulatory and anti-inflammatory effect.
- 25 This aptamer has been successfully tested in preclinical models of diseases like ischemic stroke (WO2020/230108A1) and myocardial infarction (WO2020/230109A1), therein referred to as ApTOLL (SEQ ID NO: 1), producing an excellent protective effect. In clinical studies, ApTOLL has demonstrated a very good safety profile in a completed First-in-Human clinical trial and reduced mortality compared to the placebo arm in Phase Ib/Ila trial (APRIL trial).

- 30 However, there is still the need to find new aptamers useful for treating TLR4-related diseases.

SUMMARY OF THE INVENTION

- 35 The present invention provides new aptamers that are antagonists of TLR-4 and present several advantages, as detailed below.

- Inventors have designed and synthesized six new aptamers, herein referred to as ApSION 1-6 (SEQ ID NO: 1-6) (see EXAMPLE 1 and FIG. 32), that include between 17 and 30 chemically modified
- 40 nucleoside and/or nucleotides, including nucleosides with a sugar modification and reverse

nucleoside derivatives. Particularly, the chemical modifications introduced in ApSION 1-6 were the following: 2'-fluoro-2'-deoxynucleoside (2'-F-RNA), 2'-O-methoxyethyl-nucleoside (2'-O-MOE-RNA), 2'-O-methylnucleoside (2'-O-methyl-RNA), 2'-O, 4'-C-methylene- β -D-ribofuranosyl nucleoside (LNA or locked nucleic acid), and inverted nucleosides.

5

These modifications are located in some of the following residues of the aptamers: 3, 5, 7, 8, 11, 15, 16, 18, 20, 21, 23, 24, 26, 28, 29, 32, 34, 36, 38, 39, 40, 43, 44, 47, 48, 49, 50, 54, 56 and 60 with respect to reference sequence SEQ ID NO: 7. See FIG. 32.

10 Particularly, the aptamers can comprise the same type of chemical modification throughout the entire sequence. As a way of illustration, ApSION 2 (SEQ ID NO: 2) comprises 2'-O-methoxyethyl-nucleosides in all the thymine and cytosine nucleosides, i.e., 2'-O-methoxyethyl-5-methyluridine (2'-O-MOE-T) and 2'-O-methoxyethyl-5-methylcytidine (2'-O-MOE-meC); while ApSION 3 (SEQ ID NO: 3) comprises 2'-O, 4'-C-methylene- β -D-ribofuranosyl nucleosides (LNA or locked nucleic acids) in all
15 the thymine and cytosine nucleosides, i.e., 2'-O, 4'-C-methylene- β -D-ribofuranosylthymine (LNA-T) and 2'-O, 4'-C-methylene- β -D-ribofuranosylcytosine (LNA-C).

ApSION 1-6 comprise an additional nucleotide at the 3' end of the sequence, particularly, a thymidinyl (3'-3') phosphate thymidine (inverted T) (ApSION 1-5 with SEQ ID NO: 1-5, respectively), or a 2'-
20 deoxycytidinyl (3'-3') phosphate 2'-deoxycytidine (inverted C) (ApSION 6 with SEQ ID NO: 6).

These aptamers were designed from the ApTOLL sequence (SEQ ID NO: 7), from which some chemical modifications were made to obtain a more stable and functional aptamer against TLR-4, which was unexpected considering that destructuring and loss of function are common when performing these types of modifications in aptamers. Indeed, inventors demonstrated in EXAMPLE 2 (FIG. 1-2) and EXAMPLE 3 (FIG. 3) that these aptamers are stable against DNA-ase (FIG. 1) and in plasma (FIG. 2-3), and have TLR-4 antagonist activity (EXAMPLE 4, FIG. 4).

Moreover, inventors found that breast tumor cell lines MDA-MB-231 and SUM 159, in both adherent
30 and mammosphere forms, overexpress the TLR-4 receptor, which is associated with metastasis and poor prognosis in several types of cancer (see EXAMPLE 5, FIG. 5 and EXAMPLE 6, FIG. 6).

Surprisingly, inventors also demonstrated that these new aptamers and ApTOLL have an effect on breast cancer cell lines (mammospheres), by inhibiting the ability of these cells to form spheres and
35 reducing the size of the spheres formed. Further, the treatment of these cell lines with the aptamers causes the reduction in TLR-4 receptor mRNA expression, indicative of the effect of the aptamers on the TLR-4 receptor (see EXAMPLE 7, FIG. 7-10). Therefore, these results are a clear evidence of the ability of the aptamers to reduce tumor progression and cause tumor shrinkage, useful for the treatment of cancer, particularly breast cancer.

40

Furthermore, inventors also demonstrated the ability of these new aptamers and ApTOLL to have a positive effect in experimental autoimmune neuritis (EAN) mouse model, a preclinical model of inflammatory autoimmune neuropathy disorder and Guillain-Barré syndrome (GBS) (see EXAMPLE 8, FIG. 11-18). Particularly, mice treated with the aptamers had a significant increase of neuromotor, neuromuscular and electrophysiological performances at day 22, compared to vehicle group, confirming the positive efficacy of the compounds on the electrophysiology impairment induced by the inflammatory neuropathy. Significant decrease of plasma TNF- α and IL-6 concentrations was also observed in aptamers treated animals confirming these positive data from a biomarker point of view. Furthermore, EXAMPLE 9 (FIG. 19-26) show the dose-response of these aptamers in EAN mouse model. These results strongly evidence the efficacy of the aptamers in inflammatory autoimmune neuropathy disorder and GBS.

Remarkably, inventors also demonstrated in EXAMPLE 10 that a variant aptamer having at least 90% sequence identity with the ApTOLL sequence (or at least 69% sequence identity considering the whole sequence) and optionally extended at the 5' and 3' ends by 1-13 or 1-4 nucleotides, respectively, maintains the functions (i.e., the capability of binding specifically to and inhibiting TLR4) of the original nucleic acid sequence, ApTOLL. SEQ ID NO: 13-21 are examples of variants. Thus, it is plausible that aptamers with at least 70% sequence identity to the ApTOLL sequence, particularly 90%, when combined with chemical modifications (e.g., the chemical modifications of the aptamers synthesized in EXAMPLE 1), will exert the same or similar effects as demonstrated in EXAMPLES 2-9. Therefore, when the ApTOLL sequence or its variants are combined with chemical modifications, the resulting aptamers are effective for the treatment of cancer and inflammatory autoimmune neuropathy disorder (e.g., GBS).

Furthermore, inventors also demonstrated in EXAMPLE 11 that ApSION 2 and ApSION 4 maintain the stability parameters after twelve and nine months of storage, respectively.

In addition, inventors also demonstrated the ability of these new aptamers and ApTOLL to have a positive effect in experimental autoimmune encephalomyelitis (EAE) mouse model, a preclinical model of multiple sclerosis (see EXAMPLE 12, FIG. 33-39).

Accordingly, a first aspect of the invention relates to an aptamer (e.g., SEQ ID NO: 1-6) comprising: (a) a polynucleotide sequence consisting of:

- i) SEQ ID NO: 7 or a fragment thereof (e.g., SEQ ID NO: 8); or
 - ii) a functionally equivalent variant having at least 70% sequence identity to SEQ ID NO: 7 or the fragment thereof (e.g., SEQ ID NO: 13-21), wherein the functionally equivalent variant:
 - has the capability of specifically binding to TLR-4 and inhibiting TLR-4 activation; and
 - has the secondary structure of SEQ ID NO: 7 or of the fragment thereof, as determined using mFold;
- (b) at least 10 chemically modified nucleotides; and

(c) optionally, the addition of at least one inverted nucleotide at the 3' and/or 5' end of the sequence; wherein the aptamer has the capability of specifically binding to TLR-4 and inhibiting TLR-4 activation. Particularly, the fragment thereof is SEQ ID NO: 8, the functionally equivalent variant has a length between 40 and 65 nucleotides, and the chemically modified nucleotides are sugar modified nucleotides.

Another aspect refers to a complex comprising an aptamer of the invention and a functional group.

In an aspect, the invention provides an aptamer or a complex as described herein for use as a medicament. The invention also encompasses the aptamers and complexes described herein for the treatment of a pathological condition or disease susceptible to amelioration by inhibition of TLR-4 receptor comprising administering a therapeutically effective amount of at least one aptamer or complex described herein.

Other aspects relate to an aptamer or complex as described herein for use in the treatment of cancer (e.g., breast cancer), or for use in the treatment of a neuromuscular or neurodegenerative disease or condition, particularly an inflammatory autoimmune neuropathy disorder (e.g., Guillain-Barré syndrome). Alternatively, the invention encompasses a method of treatment of cancer, or a method of treatment of a neuromuscular or neurodegenerative disease or condition, particularly an inflammatory autoimmune neuropathy disorder, more particularly Guillain-Barré syndrome, comprising administering to a subject in need thereof a therapeutically effective amount of an aptamer or a complex of the invention. The invention also encompasses uses and methods of the aptamers for the treatment of other TLR-4 mediated diseases, neurodegenerative diseases or autoimmune diseases, such as multiple sclerosis.

Other aspects relate to SEQ ID NO: 7 (ApTOLL) for the treatment of cancer, particularly breast cancer; for the treatment of an inflammatory autoimmune neuropathy disorder, particularly Guillain-Barré syndrome; and for the treatment of multiple sclerosis.

Other aspects refer to *in vitro* uses and methods of an aptamer or a complex as described herein for detecting TLR-4, or for inhibiting TLR-4. In another aspect, the invention relates to the use of a complex according to the invention for *in vivo* imaging of a cell, tissue or organ which express TLR-4, wherein said complex comprises a functional group.

In another aspect, the present invention relates to a pharmaceutical composition comprising an aptamer or a complex described herein (e.g., SEQ ID NO: 1-6) together with at least a pharmaceutically acceptable excipient, carrier, or solvent.

Another aspect relates to methods of making the aptamers or complexes of the present invention.

Throughout the description and claims the word "comprise" and its variations are not intended to exclude other technical features, additives, components, or steps. Additional objects, advantages and features of the invention will become apparent to those skilled in the art upon examination of the description or may be learned by practice of the invention. Furthermore, the present invention covers all possible combinations of particular and preferred embodiments described herein. The following examples and drawings are provided herein for illustrative purposes, and without intending to be limiting to the present invention.

DESCRIPTION OF DRAWINGS

- FIG. 1** shows the resistance of aptamers to degradation by λ -exonuclease and DNase I. (A) Representative gel of the stability of each aptamer in the presence of nucleases in a concentration range of 0.01-1 unit. (B) Quantification of DNase I resistance assays. Means $\pm$ S.E.M. of 3 independent experiments are plotted. * $p < 0.05$ with respect to the control (no DNase I).
- FIG. 2** shows the resistance of aptamers to degradation by human (A), rat (B) and non-human primate (NHP)(C) plasma nucleases. Bars represent the mean $\pm$ S.E.M. of 3 different plasmas. * $p < 0.05$ with respect to the control (time 0).
- FIG. 3** shows the fluorescence measurement of each fraction eluted from the sephacryl S-200 column after resolving 200 pmoles of aptamer previously incubated with 150 μ L of plasma 30 min at 37 $^{\circ}$ C. The shaded region corresponds to the aptamer elution peak. Human plasmas incubated with ApSION 2 (A) and ApSION 4 (D); rat plasmas incubated with ApSION 2 (B) and ApSION 4 (E); non-human primate (NHP) plasmas incubated with ApSION 2 (C) and ApSION 4 (F).
- FIG. 4** shows (A) TLR-2 antagonist activity assay-ApSIONs, (B) TLR-4 antagonist activity assay-ApSIONs, and (C) TLR-5 antagonist activity assay-ApSIONs.
- FIG. 5** shows the analysis of TLR-4 mRNA levels in HEK-293T, MDA-MB-231, and SUM159 cells. Bars correspond to the mean $\pm$ SEM of 3 independent experiments.
- FIG. 6** shows the TLR-4 mRNA expression levels in MDA-MB-231 and SUM159 cells as adherent cells and mammospheres. Cells were seeded on p24 plates at a density of 50,000 cells/poc for adherent cells for 24h, and 5000 cells/poc on p24 ULA (ultra-low attachment) plates for mammospheres for 15 days. RNA was then extracted, and TLR-4 messenger RNA expression was measured by real-time qPCR (RT-qPCR). Bars correspond to the mean $\pm$ SEM of 3 independent experiments.

FIG. 7 shows the effect of aptamers on the number and size (diameter and perimeter) of mammospheres obtained from SUM159 triple-negative breast cancer cells. Bars represent the mean $\pm$ SEM of 2-3 independent experiments.

- 5 **FIG. 8** shows the effect of aptamers on TLR-4 mRNA levels. The bars represent the mean $\pm$ SEM of 2-3 independent experiments. *, $p < 0.05$ with respect to 1.

- FIG. 9** shows the effect of aptamers on the number and size of mammospheres obtained from SUM159 (A) and MDA-MB-231 (B) triple-negative breast cancer cells. Bars represent mean $\pm$ SEM
10 of 3-5 independent experiments. *, $p < 0.05$; **, $p < 0.01$ with respect to control.

FIG. 10 shows the effect of aptamers on TLR-4 mRNA levels. Bars represent mean $\pm$ SEM of 2-3 independent experiments. *, $p < 0.05$, **, $p < 0.001$ with respect to 1.

- 15 **FIG. 11** shows the study scheme of the study in EAN mouse models.

FIG. 12 is the graphical representation of animal body weight. * marks statistical differences over sham (negative control) group; # marks statistical differences over GBS + vehicle group.

- 20 **FIG. 13** is the graphical representation of rotarod latency at the baseline (day 1) and day 22. * marks statistical differences over sham (negative control) group; # marks statistical differences over GBS + vehicle group.

- FIG. 14** is the graphical representation of grip strength at the baseline (day 1) and day 22. * marks
25 statistical differences over sham (negative control) group; # marks statistical differences over GBS + vehicle group.

- FIG. 15** is the graphical representation of CMAP amplitude at the baseline (day 1) and day 22. * marks statistical differences over sham (negative control) group; # marks statistical differences over
30 GBS + vehicle group.

- FIG. 16** is a graphical representation of the nerve conduction velocity at the baseline (day 1) and day 22. * marks statistical differences over sham (negative control) group; # marks statistical differences
over GBS + vehicle group.

- 35 **FIG. 17** is a graphical representation of plasma TNF- α concentration at day 22 in infold over sham (negative control) group. * marks statistical differences over sham (negative control) group; # marks statistical differences over GBS + vehicle group.

FIG. 18 is a graphical representation of plasma IL-6 concentration at day 22 in infold over sham (negative control) group. * marks statistical differences over sham (negative control) group; # marks statistical differences over GBS + vehicle group.

5 **FIG. 19** is the graphical representation of animal body weight in the dose-response efficacy study (GBS + vehicle; GBS + ApS10N 2 at different doses; and GBS + ApS10N 4 at different doses). Tested doses: GBS + ApS10N 2 at 0.23 mg/kg, 0.45 mg/kg, 0.9 mg/kg, 1.8 mg/kg and 3.6 mg/kg; and GBS + ApS10N 4 at 0.23 mg/kg, 0.45 mg/kg, 0.9 mg/kg, 1.8 mg/kg and 3.6 mg/kg. * marks statistical differences over GBS + vehicle group.

10

FIG. 20 is the graphical representation of BBB motor disability scoring at day 10, 16 and 22 in the dose-response efficacy study (GBS + vehicle; GBS + ApS10N 2 at different doses; and GBS + ApS10N 4 at different doses). * marks statistical differences over GBS + vehicle group.

15 **FIG. 21** is the graphical representation of rotarod latency at the baseline (day 1) and day 22 in the dose-response efficacy study (GBS + vehicle; GBS + ApS10N 2 at different doses; and GBS + ApS10N 4 at different doses). * marks statistical differences over GBS + vehicle group.

20 **FIG. 22** is the graphical representation of grip strength at the baseline (day 1) and day 22 in the dose-response efficacy study (GBS + vehicle; GBS + ApS10N 2 at different doses; and GBS + ApS10N 4 at different doses). * marks statistical differences over GBS + vehicle group.

25 **FIG. 23** is the graphical representation of CMAP amplitude at the baseline (day 1) and day 22 in the dose-response efficacy study (GBS + vehicle; GBS + ApS10N 2 at different doses; and GBS + ApS10N 4 at different doses). * marks statistical differences over GBS + vehicle group.

FIG. 24 is a graphical representation of the nerve conduction velocity at the baseline (day 1) and day 22 in the dose-response efficacy study (GBS + vehicle; GBS + ApS10N 2 at different doses; and GBS + ApS10N 4 at different doses). * marks statistical differences over GBS + vehicle group.

30

FIG. 25 is a graphical representation of plasma TNF- α concentration at day 22 in infold over vehicle group in the dose-response efficacy study (GBS + vehicle; GBS + ApS10N 2 at different doses; and GBS + ApS10N 4 at different doses). * marks statistical differences over GBS + vehicle group.

35 **FIG. 26** is a graphical representation of plasma IL-6 concentration at day 22 in infold over vehicle group in the dose-response efficacy study (GBS + vehicle; GBS + ApS10N 2 at different doses; and GBS + ApS10N 4 at different doses). * marks statistical differences over GBS + vehicle group.

40 **FIG. 27** represents the antagonist TLR4 activity assay of ApTOLL (SEQ ID NO: 7) and its variants ApTOLL-Mut 1-6 (SEQ ID NO: 13-18). The activity of TLR4 receptor is represented in percentage

with respect to control LPS-Ek up. The antagonist activity of the aptamer is identified by the decrease of activation percentage in regard to LPS-EK up.

5 **FIG. 28** represents the competition assay for TLR4 receptor of ApTOLL (SEQ ID NO: 7) and its variants ApTOLL-Mut 1-6 (SEQ ID NO: 13-18). The mutants competition with ApTOLL sequence for the same binding site in TLR4 receptor is identified by decrease of binding percentage of ApTOLL sequence (control) in each mix ApTOLL/ApTOLL-Mut (1-6) in respect to ApTOLL.

10 **FIG. 29** depicts the correlation between the secondary structure of ApTOLL (SEQ ID NO: 7) and the position of additions, substitutions or deletions in variants with about 90% sequence identity to ApTOLL sequence, i.e., SEQ ID NO: 13-18. These variants can have additions, substitutions or deletions in stems (A), in the connector (B) or in loops (C).

15 **FIG. 30** illustrates the correlation between the secondary structure of ApTOLL (SEQ ID NO: 7) and the position of additions at the ends (i.e., extensions at either the 5' or 3' ends) of variants with about 77.6% sequence identity to ApTOLL sequence, e.g., SEQ ID NO: 19.

20 **FIG. 31** shows the stability parameters (purity, impurity profile, total impurity and biological activity) after twelve months for ApSION 2 (A) and nine months for ApSION 4 (B) of storage.

25 **FIG. 32** illustrates the pattern of chemically modifications along the sequence of the new aptamers ApSION 1-6 (SEQ ID NO: 1-6), compared to ApTOLL sequence (SEQ ID NO: 7). The shaded bases represent those that are chemically modified (in this illustration the type of chemical modification is not distinguished). Additionally, ApSION 1-5 have an additional 3' inverted T, and ApSION 6 has an additional 3' inverted C, compared to the ApTOLL sequence.

30 **FIG. 33** shows the dose-response study for ApSION 2 and ApSION 4 in the murine EAE model of MS. (A) Evolution of the clinical score after injection of ApSION 2 at different doses. (B) Clinical score development after injection of ApSION 4 at different doses. The number of animals used is indicated in parentheses next to the name of each experimental group. The results of the Student's t-test after comparing the EAE-Veh group with each of the other experimental groups at each post-onset day are represented in the individual tables as follows: *p<0,05; **p<0,01; ***p<0,001 for comparisons of ApSION and ApTOLL with vehicle; #p<0.05; ; ##p<0,01; ####p<0,001 for comparisons of ApSION and ApTOLL. Abbreviations: n.s = non significative.

35 **FIG. 34** shows the analysis of demyelinating lesions. (A) Representative images of spinal cord sections after eriochrome-cyanine staining. (B) Quantification of the percentage of demyelinated area within the white matter of each experimental group. (C) Comparison between ApSION 2 (0.45 mg/Kg and 0.91 mg/Kg) and ApTOLL (0.91 mg/Kg) of demyelination ratio with respect to the vehicle group.

The results of the of the Student's t-test (B) and one-way ANOVA test for multiple comparisons (C) are represented as: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

FIG. 35 shows the histological analysis of myelin. (A) Representative images of spinal cord sections stained with MBP marker. (B) Quantification of the percentage of MBP area within the white matter of each experimental group. (C) Comparison between ApSION 2 (0.45 mg/Kg and 0.91 mg/Kg) and ApTOLL (0.91 mg/Kg) of MBP ratio with respect to the vehicle group. The results of the of the Student's t-test (B) and one-way ANOVA test for multiple comparisons (C) are represented as: * $p < 0.05$; ** $p < 0.01$.

10

FIG. 36 shows the histological analysis of neurofilaments. (A) Representative images of spinal cord sections stained with NFH marker. (B) Quantification of the percentage of NFH area within the white matter of each experimental group. (C) Comparison between ApSION 2 (0.45 mg/Kg and 0.91 mg/Kg) and ApTOLL (0.91 mg/Kg) of NFH ratio with respect to the vehicle group. The results of the of the Student's t-test (B) and one-way ANOVA test for multiple comparisons (C) are represented as: * $p < 0.05$; ** $p < 0.01$.

FIG. 37 shows the oligodendroglial lineage analysis. (A) Magnified images of oligodendrocytes (labeled with Olig2) in the spinal cord. (B) Quantification of the percentage of Olig2⁺ cells out of total number of cells of each experimental group. (C) Comparison between ApSION 2 (0.45 mg/Kg and 0.91 mg/Kg) and ApTOLL (0.91 mg/Kg) of Olig2⁺ cells with respect to the vehicle group. The results of the of the Student's t-test (B) and one-way ANOVA test for multiple comparisons (C) are represented as: * $p < 0.05$; ** $p < 0.01$.

FIG. 38 shows the oligodendrocyte precursor cells (OPCs) analysis. (A) Magnified images of OPCs (labeled with PDGFR α) in the spinal cord. (B) Quantification of the percentage of PDGFR α *Olig2⁺ cells with respect the total number of Olig2⁺ cells of each experimental group. (C) Comparison between ApSION 2 (0.45 mg/Kg and 0.91 mg/Kg) and ApTOLL (0.91 mg/Kg) of OPCs with respect to the vehicle group. The results of the + Student's t-test (B) and one-way ANOVA test for multiple comparisons (C) are represented as: * $p < 0.05$.

FIG. 39 shows the cytokine array analysis. (A) Cytokine/chemokine array results from EAE animals treated with ApTOLL and vehicle. The number of animals included in each group was 6. (B) Cytokine/chemokine array results from EAE animals treated with ApSION 2 and vehicle. Both tables (A) and (B) indicate the values of integrated density (mean $\pm$ SEM) of each mouse for the different molecules. (C) Cytokine array results from EAE animals treated with ApSION 2 (0.45 mg/Kg) and vehicle. The graph indicates the molecules that showed differences between the two groups. Two animals per experimental group were used. Student's t-test for comparing pairs of groups is represented as * $p < 0.05$.

40

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to chemically modified aptamers derived from SEQ ID NO: 7 or its variants. Also provided are uses and methods of treatment of a disease (e.g., breast cancer or an inflammatory autoimmune neuropathy); pharmaceutical compositions and formulations comprising aptamers; and methods of manufacture and of formulation.

Before the present invention is described in greater detail, it is to be understood that this invention is not limited to the particular compositions or process steps described, as such can, of course, vary. As will be apparent to those skilled in the art upon reading this description, each of the individual embodiments described and illustrated herein has discrete components and features which can be readily separated from or combined with the features of any of the other several embodiments without departing from the scope or spirit of the present description. Any recited method can be carried out in the order of events recited or in any other order which is logically possible.

The headings provided herein are not limitations of the various embodiments of the description, which can be defined by reference to the specification as a whole. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present description will be limited only by the appended claims.

Accordingly, the terms defined immediately below are more fully defined by reference to the description in its entirety.

Definitions

In order that the present description can be more readily understood, certain terms are first defined. Additional definitions are set forth throughout the detailed description.

Aptamer: this term, in the context of the present invention, refers to single-stranded nucleic acid chains adopting a specific tertiary structure that allows them to bind to molecular targets with high specificity and affinity, comparable to that of monoclonal antibodies, through interactions other than conventional Watson-Crick base pairing. Generally, aptamers are selected from combinatorial libraries by systemic evolution of ligands by exponential enrichment (SELEX) technology. SELEX is used to identify DNA and RNA aptamers that recognize and selectively bind extra- and intracellular target molecules with high specificity and nanomolar affinity. Once folded under physiological conditions, aptamers acquire unique three-dimensional structures based on their nucleotide sequence, being the tertiary structure of aptamers that confers the selectivity and affinity for their targets.

The term "aptamer of the present invention" or "the aptamer disclosed herein" and grammatical

variants thereof refers to an aptamer that specifically binds to an epitope located on the extracellular domain of TLR-4, e.g., act as a TLR-4 antagonist. In some embodiments, the aptamers of the present invention block the inflammatory response released after the onset of a disease or condition disclosed herein (e.g., breast cancer, an inflammatory autoimmune neuropathy or Guillain-Barré syndrome). Non-limiting examples of aptamers are ApSION 1-6 (SEQ ID NO: 1-6), ApTOLL (SEQ ID NO: 7), a fragment of ApTOLL (e.g., SEQ ID NO: 8), or a functionally equivalent variant of ApTOLL (e.g., SEQ ID NO: 13-22). In some embodiments, the aptamer of the present invention is an aptamer of SEQ ID NO: 1-6, that are chemically modified, or a chemically modified aptamer derived from SEQ ID NO: 7-8 or 13-22. In the present invention, the aptamers are polynucleotides or nucleic acid aptamers (see definitions below).

Binding specificity: According to the art and as understood by the skilled person - the terms "specificity" or "binding specificity" or "specifically binding" refer to the ability of a binding molecule, e.g., an aptamer of the present disclosure, to bind preferentially to an epitope versus a different epitope and does not necessarily imply high affinity. The terms "binding specificity" and "specificity" are used interchangeably and can refer both to (i) a specific portion of a binding molecule (e.g., an aptamer), and (ii) the ability of the binding molecule to specifically bind to a particular epitope. A binding molecule, e.g., an aptamer, "specifically binds" when there is a specific interaction between the aptamer and its target epitope. The term "specifically binds" means that the aptamer has been generated to bind to its target epitope. The term "non-specific binding" means that an aptamer has not been generated to specifically bind to a target epitope but does somehow bind to the epitope through non-specific means.

Nucleic acid: "nucleic acid," "nucleic acid molecule", "nucleotide sequence", "polynucleotide", and grammatical variants thereof are used interchangeably and refer to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine; "RNA molecules") or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; "DNA molecules"), or any phosphoester analogs thereof, such as phosphorothioates and thioesters, in either single stranded form, or a double-stranded helix.

Single stranded nucleic acid sequences refer to single-stranded DNA (ssDNA) or single-stranded RNA (ssRNA). Double stranded DNA-DNA, DNA-RNA and RNA-RNA helices are possible. The term nucleic acid molecule, and in particular DNA or RNA molecule, refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear or circular DNA molecules (e.g., restriction fragments), plasmids, supercoiled DNA and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences can be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the non-transcribed strand of DNA (i.e., the strand having a sequence homologous to the mRNA). DNA includes, but is not limited to, cDNA, genomic DNA, plasmid DNA, synthetic DNA, and semi-synthetic DNA.

In some embodiments, a polynucleotide can be, e.g., an aptamer of the present invention (e.g., SEQ ID NO: 1-6). In some embodiments, the polynucleotide is a DNA. In some embodiments, the DNA is a synthetic DNA, e.g., a synthetic ssDNA. In some embodiments, the synthetic DNA comprises at least one chemical modification, such as nucleoside analogues having chemically modified bases or sugars, modifications of the backbone, etc.

Antagonist: as used herein, the term "antagonist" refers to a molecule that blocks or dampens an agonist mediated response rather than provoking a biological response itself upon bind to a receptor. Many antagonists achieve their potency by competing with endogenous ligands or substrates at structurally defined binding sites on the receptors. An antagonist can be a competitive, non-competitive, or uncompetitive antagonist. In some embodiments of the present invention, the antagonist is a TLR-4 antagonist, e.g., aptamers such as SEQ ID NO: 1-7 and SEQ ID NO: 13-21.

TLR-4: this term as used herein refers to membrane receptor toll-like receptor 4. Receptor TLR-4 can also be referred to as ARMD10, CD284, TLR-4 or hTOLL. In humans, receptor TLR-4 was registered in GenBank under accession number O00206.2 on 27th May, 2014, and it is encoded by the *TLR4* gene. It is made up of 839 amino acids, of which residues 1-23 constitute the signal sequence, residues 24-631 constitute the extracellular domain, residues 632-652 constitute the transmembrane domain, and residues 653-839 constitute the cytoplasmic domain.

In a particular embodiment, the aptamers disclosed herein can bind specifically to the extracellular domain of TLR-4 (amino acids 24-631).

Nucleotide: this term as used herein refers to the monomers making up the nucleic acids. The nucleotides are formed by a pentose (or sugar), a nitrogenous base and a phosphate group, and are bound by means of phosphodiester bonds. The nucleotides that are part of DNA and RNA differ in the pentose, this being deoxyribose and ribose, respectively. The nitrogenous bases, in turn, are divided into purine nitrogenous bases, which are adenine (A) and guanine (G), and into pyrimidine nitrogenous bases, which are thymine (T), cytosine (C) and uracil (U).

Identity: this term as used herein refers to the overall monomer conservation between polymeric molecules, e.g., between polynucleotide molecules (e.g. DNA and/or RNA molecules). The term "identical" without any additional qualifiers, e.g., nucleic acid A is identical to nucleic acid B, implies the sequences are 100% identical (100% sequence identity). Describing two sequences as, e.g., "70% identical", is equivalent to describing them as having, e.g., "70% sequence identity".

Calculation of the percent identity of two polymeric molecules, e.g., polynucleotide sequences, can be performed, e.g., by aligning the two sequences for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second polynucleotide sequences for optimal alignment

and non-identical sequences can be disregarded for comparison purposes). In certain embodiments, the length of a sequence aligned for comparison purposes is at least about: 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% of the length of the reference sequence. The bases at corresponding base positions, in the case of polynucleotides, are then compared.

When a position in the first sequence is occupied by the same base as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which needs to be introduced for optimal alignment of the two sequences. The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm.

Suitable software programs are available from various sources, and for alignment of both protein and nucleotide sequences. One suitable program to determine percent sequence identity is bi2seq, part of the BLAST suite of program available from the U.S. government's National Center for Biotechnology Information BLAST web site. Bi2seq performs a comparison between two sequences using either the BLASTN or BLASTP algorithm. BLASTN is used to compare nucleic acid sequences, while BLASTP is used to compare amino acid sequences. Other suitable programs are, e.g., Needle, Stretcher, Water, or Matcher, part of the EMBOSS suite of bioinformatics programs and also available from the European Bioinformatics Institute (EBI). Sequence alignments can be conducted using methods known in the art such as MAFFT, Clustal (ClustalW, Clustal X or Clustal Omega), MUSCLE, etc. Needleman-Wunsch algorithm, among others, can be used for a global sequence alignment.

Different regions within a single polynucleotide or polypeptide target sequence that aligns with a polynucleotide or polypeptide reference sequence can each have their own percent sequence identity. It is noted that the percent sequence identity value is rounded to the nearest tenth. For example, 80.11, 80.12, 80.13, and 80.14 are rounded down to 80.1, while 80.15, 80.16, 80.17, 80.18, and 80.19 are rounded up to 80.2. It also is noted that the length value will always be an integer.

In certain embodiments, the percentage identity (%ID) of a first amino acid sequence or nucleic acid sequence to a second amino acid sequence or nucleic acid sequence is calculated as $\%ID = 100 \times (Y/Z)$, where Y is the number of amino acid residues or nucleobases scored as identical matches in the alignment of the first and second sequences (e.g., as aligned by visual inspection or a particular sequence alignment program) and Z is the total number of residues in the second sequence. If the length of a first sequence is longer than the second sequence, the percent identity of the first sequence to the second sequence will be higher than the percent identity of the second sequence to the first sequence.

One skilled in the art will appreciate that the generation of a sequence alignment for the calculation

of a percent sequence identity is not limited to binary sequence-sequence comparisons exclusively driven by primary sequence data. It will also be appreciated that sequence alignments can be generated by integrating sequence data with data from heterogeneous sources such as structural data (e.g., crystallographic protein structures), functional data (e.g., location of mutations), or phylogenetic data. A suitable program that integrates heterogeneous data to generate a multiple sequence alignment is T-Coffee. It will also be appreciated that the final alignment used to calculate percent sequence identity can be curated either automatically or manually.

In some embodiments, a polynucleotide (e.g., an aptamer) has a sequence identity with respect of one of the aptamers (e.g., SEQ ID NO: 1-6) of at least about: 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95%, wherein the nucleotide sequence maintains the has the capability of specifically binding to TLR-4 and inhibiting TLR-4 activation.

Chemical modification: in the context of nucleic acids, this term as used herein refers to the covalent alteration of the nucleotides or nucleosides of a nucleic acid molecule, typically by adding a functional group to the sugar, nucleotide base, or phosphate backbone. These modifications can alter the physical and chemical properties of the nucleic acid, and affect its function and properties. The term "chemical modification", "chemically modified", "chemically modified aptamer", "chemically modified nucleosides and/or nucleotides", or any grammatical variant thereof, can be used interchangeably in this description.

Treatment: The terms "treat", "treatment", "therapy", as used herein refers to a clinical intervention to prevent (e.g., suppress or inhibit) a disease or condition; cure the disease or condition; delay the onset of the disease or condition (e.g., breast cancer or an inflammatory autoimmune neuropathy); reduce the seriousness or severity of the disease or condition; ameliorate or eliminate one or more symptoms or sequelae associated with a disease or condition; or the provision of beneficial effects to a subject with a disease or condition, without necessarily curing the disease or condition.

In some embodiments, the term refers to a clinical intervention to improve one or more symptoms; improve one or more sequelae; prevent (e.g., suppress, inhibit or delay) one or more symptoms; prevent (e.g., suppress, inhibit or delay) one or more sequelae; delay one or more symptoms; delay one or more sequelae; ameliorate one or more symptoms; ameliorate one or more sequelae; shorten the duration one or more symptoms; shorten the duration of one or more sequelae; reduce the frequency of one or more symptoms; reduce the frequency of one or more sequelae; reduce the severity of one or more symptoms; reduce the severity of one or more sequelae; improve the quality of life; increase survival; prevent (e.g., suppress, inhibit or delay) a recurrence of the disease or condition; delay a recurrence of the disease or condition; reduce the severity of the disease (e.g., reduce the inflammation of Guillain-Barré syndrome); or any combination thereof, e.g., with respect to what is expected in the absence of the treatment with the aptamers of the present invention.

40

The term "treatment" also includes prophylaxis or prevention (e.g., suppression, inhibition or delay) of a disease or condition or its symptoms or sequelae thereof. Prophylaxis refers to a therapeutic or course of action used to prevent, inhibit, suppress, reduce the risk, reduce the occurrence or delay the onset of a disease or condition, or to prevent, inhibit, suppress, or delay a symptom associated with a disease or condition.

In some embodiments, the disease or condition is breast cancer or an inflammatory autoimmune neuropathy (e.g., Guillain-Barré syndrome).

Subject: The terms "subject", "patient", and "individual", and variants thereof are used interchangeably herein and refer to any mammalian subject, including without limitation, humans, domestic animals (e.g., dogs, cats and the like), farm animals (e.g., cows, sheep, pigs, horses and the like), and laboratory animals (e.g., monkey, rats, mice, rabbits, guinea pigs and the like) for whom diagnosis, treatment, or therapy is desired, particularly humans. The uses and methods described herein are applicable to both human therapy and veterinary applications. In an embodiment, the subject is a human.

About: The term "about" is used herein to mean approximately, roughly, around, or in the regions of. When the term "about" is used in conjunction with a numerical range, it modifies that range by extending the boundaries above and below the numerical values set forth. In general, the term "about" can modify a numerical value above and below the stated value by a variance of, e.g., 10 percent, up or down (higher or lower). As used herein, the terms "about" or "at least about" when applied to a series of values or range, apply equally to all member of the list. Accordingly, "at least about 1, 2, 3, 4..." would be interchangeable with "at least about 1, at least about 2, at least about 3, at least about 4...".

Chemically modified aptamer

The present invention is directed to chemically modified nucleic acid aptamers (e.g., SEQ ID NO: 1-6) derived from SEQ ID NO: 7 (i.e., ApTOLL sequence) or its variants with improved stability and new therapeutic effects.

Accordingly, an aspect of the present invention relates to an aptamer comprising:

- (a) a polynucleotide sequence consisting of:
- i) SEQ ID NO: 7; or
 - ii) a functionally equivalent variant having at least 70% sequence identity to SEQ ID NO: 7, wherein the functionally equivalent variant:
 - has the capability of specifically binding to TLR-4 and inhibiting TLR-4 activation; and
 - has the secondary structure of SEQ ID NO: 7 as determined using mFold;
- (b) at least 10 chemically modified nucleotides; and

(c) optionally, the addition of at least one inverted nucleotide at the 3' and/or 5' end of the sequence; wherein the aptamer has the capability of specifically binding to TLR-4 and inhibiting TLR-4 activation.

- 5 Alternatively, an aspect of the present invention relates to an aptamer comprising:
- (a) a polynucleotide sequence consisting of:
- i) SEQ ID NO: 7 or a fragment thereof (e.g., SEQ ID NO: 8); or
 - ii) a functionally equivalent variant having at least 70% sequence identity to SEQ ID NO: 7 or the fragment thereof, wherein the functionally equivalent variant:
- 10 - has the capability of specifically binding to TLR-4 and inhibiting TLR-4 activation; and
- has the secondary structure of SEQ ID NO: 7 or of the fragment thereof, as determined using mFold;
- (b) at least 10 chemically modified nucleotides; and
- (c) optionally, the addition of at least one inverted nucleotide at the 3' and/or 5' end of the sequence;
- 15 wherein the aptamer has the capability of specifically binding to TLR-4 and inhibiting TLR-4 activation.

Another aspect of the present invention relates to an aptamer comprising:

- (a) the polynucleotide SEQ ID NO: 7 or a fragment thereof, comprising at least 10 chemically modified nucleotides, wherein the aptamer has a length between 40 and 100 nucleotides and has the capability of specifically binding to TLR-4 and inhibiting TLR-4 activation; or
- 20 (b) a functionally equivalent variant having at least 70% sequence identity to the aptamer of (a), wherein the functionally equivalent variant maintains the capability of specifically binding to TLR-4 and inhibiting TLR-4 activation.

- 25 The fragment of SEQ ID NO: 7 refers to a shortened polynucleotide contained in SEQ ID NO: 7. In some embodiments, the fragment of SEQ ID NO: 7 has a length between 35 and 55 nucleotides, between 35 and 50 nucleotides, particularly between 40 and 45 nucleotides. More particularly, the fragment has a length of 41 nucleotides. In some embodiments, the fragment of SEQ ID NO: 7 is a polynucleotide contained between nucleotides 5 and 55 of SEQ ID NO: 7, particularly between nucleotides 5 and 50, and more particularly between nucleotides 7 and 46 (i.e., SEQ ID NO: 8). In a particular embodiment, the fragment of SEQ ID NO: 7 is SEQ ID NO: 8.
- 30

- For the sake of clarity, the aptamers of the present invention are chemically modified with respect to an aptamer without any modification, which can be named the reference or control aptamer, which can be SEQ ID NO: 7, a fragment thereof (e.g., SEQ ID NO: 8), or a variant thereof (e.g., SEQ ID NO: 13-22). Therefore, when referring to the incorporation of chemical modifications into an aptamer, reference is made to the introduction of modifications to a reference aptamer (e.g., SEQ ID NO: 7-8, 13-22); and when referring to aptamers that include/comprise/present some chemical modification,
- 35
- 40 reference is made to the aptamers of the present invention (e.g., SEQ ID NO: 1-6).

The term "nucleotide" as used herein refers to both terms "nucleotide" and "nucleoside". A "nucleoside" refers to a compound containing a sugar molecule (e.g., a pentose or ribose) or a derivative thereof in combination with an organic base (e.g., a purine or pyrimidine) or a derivative thereof (also referred to herein as "nucleobase"). A "nucleotide" refers to a nucleoside including a phosphate group. Since a "nucleotide" includes a "nucleoside", the terms "nucleotide" and "nucleoside" are used indistinctly in this description in order to simplify the reading. Nucleotide/nucleoside will also be used hereinafter.

10 In some embodiments, the aptamer has a length between 40 and 100 nucleotides, between 40 and 80 nucleotides, particularly between 40 and 65, and more particularly between 40 and 60, or between 42 and 60. In specific embodiments, the aptamer has a length of 60 nucleotides (e.g., ApSION 1-5, SEQ ID NO: 1-5), or a length of 42 nucleotides (e.g., ApSION 6, SEQ ID NO: 6).

15 The ability of an aptamer of the present invention (e.g., SEQ ID NO: 1-6) of specifically bind to TLR-4 can be determined, e.g., by *in vitro* binding assays, such as the enzyme-linked oligonucleotide assay (ELONA), the enzyme-linked aptamer sorbent assay (ELASA), precipitation and quantitative PCR (qPCR), or by fluorescence techniques such as aptahistochemistry, aptacytochemistry, fluorescence microscopy or flow cytometry. Likewise, both the capability of specific binding to TLR-4 and
20 the affinity of the aptamer for TLR-4 can be determined by techniques well-known by the person skilled in the art, such as gel mobility shift assay, surface plasmon resonance (SPR), kinetic capillary electrophoresis and fluorescence binding assay. Briefly, the fluorescence binding assay consists of the incubation of magnetic balls coated with TLR-4 with different concentrations (e.g., from 0 to 100 nM) of the aptamer of the invention labeled (e.g., with carboxyfluorescein, FAM), and the subsequent
25 elution and detection of the bound aptamers; the dissociation constants (K_d) are calculated by non-linear fit analysis.

The terms "inhibit TLR-4," "inhibition of TLR-4", "TLR-4 inhibition " and grammatical variants thereof refer to the blocking and/or reduction of the activation and/or activity of TLR-4, e.g., the transduction
30 of the TLR-4-mediated signal. In the context of the present invention, it is considered that TLR-4 is inhibited by an aptamer of the present invention (e.g., SEQ ID NO: 1-6) if the signaling activity of TLR-4 is reduced by at least about: 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% compared to the activity of TLR-4 in the present of a natural agonists, e.g., lipopolysaccharide (LPS). Lipopolysaccharide, also known as
35 endotoxin, is a major glycolipid constituent of the outer cell wall of gram-negative bacteria. LPS molecules typically consist of a strain-specific distal polysaccharide side chain known as the O-antigen, a hydrophilic core oligosaccharide, and a hydrophobic domain referred to as lipid A. In some embodiments, the term "inhibit TLR-4" refers, e.g., to (i) blockage or complete inhibition of TLR-4 activation, (ii) reduction or partial inhibition of TLR-4 activation, (iii) blockage or complete inhibition of TLR-4
40 signaling activity, (iv) reduction or partial inhibition of TLR-4 signaling activity, or (v) any combination

thereof, by the aptamers of the present invention. In this description, "inhibition" and "reduction" of TLR-4 activation can be used interchangeably.

5 The ability of an aptamer of the present invention (e.g., SEQ ID NO: 1-6) to inhibit TLR-4 can be determined by means of a range of assays available in the art. In some embodiments, the capability of inhibiting TLR-4 of an aptamer is determined by means of *in vitro* assays with cells expressing recombinant TLR-4 and a reporter gene, the expression of which is associated with the activation of recombinant TLR-4. The person skilled in the art will recognize that there are multiple variants of this method, depending on the cell and the recombinant gene used. An example of this assay is included
10 e.g., in U.S. Pat. No. 10,196,642, which is herein incorporated by reference in its entirety. Other available techniques include the determination of the levels of inflammatory cytokines, such as IL-1, IL-8, TNF-alpha and IL-12, released by cells that express TLR-4. An example of an assay is included in the examples provided herein (see EXAMPLE 4).

15 To determine the secondary structure of the aptamer using mFold, a computational tool widely recognized for predicting RNA secondary structures, the nucleotide sequence of the aptamer is input into the software. mFold employs algorithms grounded in thermodynamics and minimum free energy principles to forecast the most thermodynamically stable secondary structure of the RNA molecule. Parameters such as ionic conditions, temperature, and any pertinent experimental constraints or
20 modifications are set to mirror actual experimental conditions closely. Subsequently, the software generates various output files, including dot plots, structure diagrams, and energy calculations, each representing potential secondary structures and their respective stabilities. The most energetically favorable or biologically pertinent structure is then selected based on criteria such as thermodynamic stability, conservation across related sequences, and experimental validation, if available. This predicted secondary structure is then juxtaposed with the known secondary structure of the reference aptamer to ensure conformity in structural features, such as stem-loop motifs and overall folding topology. Experimental techniques like enzymatic probing or chemical mapping can be deployed to corroborate the predicted secondary structure and ascertain its alignment with the reference aptamer (e.g., SEQ ID NO: 7).

30

Functionally equivalent variants

As said, the aptamer provided herein comprises a polynucleotide sequence consisting of (i) SEQ ID NO: 7 (or a fragment thereof); or (ii) a functionally equivalent variant having at least 70% sequence
35 identity to SEQ ID NO: 7 or the fragment thereof, wherein the functionally equivalent variant: has the capability of specifically binding to TLR-4 and inhibiting TLR-4 activation; and has the secondary structure of SEQ ID NO: 7 or of the fragment, as determined using mFold. This aptamer is subjected to chemical modifications as explained.

These variants can include in their polynucleotide sequence, additions at both the 5' and 3' ends, internal deletions, conservative or non-conservative substitutions, while maintaining their biological activity as demonstrated in EXAMPLE 9 (e.g., SEQ ID NO: 13-21).

- 5 In some embodiments, the functionally equivalent variant is a polynucleotide sequence with a sequence identity of at least: 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% with SEQ ID NO: 7. In a particular embodiment, the functionally equivalent variant is a polynucleotide sequence with a sequence identity of at least 90% with SEQ ID NO: 7. In another particular
- 10 embodiment, the functionally equivalent variant is a polynucleotide sequence with a sequence identity of at least about 77% with SEQ ID NO: 7. In another particular embodiment, the functionally equivalent variant is a polynucleotide sequence with a sequence identity of at least about 69% with SEQ ID NO: 7.
- 15 In an embodiment, the functionally equivalent variant is selected from the group consisting of SEQ ID NO: 13-22. In another embodiment, the functionally equivalent variant is selected from the group consisting of SEQ ID NO: 13-21. In a particular embodiment, the functionally equivalent variant is selected from the group consisting of SEQ ID NO: 13-18, i.e., said variant having a sequence identity of at least about 90% with SEQ ID NO: 7. In another embodiment, the functionally equivalent variant
- 20 is SEQ ID NO: 19, i.e., said variant having a sequence identity of at least about 77% with SEQ ID NO: 7. In another embodiment, the functionally equivalent variant is SEQ ID NO: 20 or 21, i.e., said variant having a sequence identity of at least about 69% with SEQ ID NO: 7.

- 25 In some embodiments, the functionally equivalent variant includes sequences with the addition, deletion, or substitution of: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides with respect to sequence SEQ ID NO: 7. The addition, deletion, or substitution can be located at any nucleotide of SEQ ID NO: 7, and particularly at the 3' and/or 5' ends.

- 30 In some embodiments, the functionally equivalent variant includes a sequence with extensions, i.e., the addition of: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides, particularly at the 3' and/or 5' ends of the sequence SEQ ID NO: 7, particularly between 1 and 15 nucleotides.

- 35 FIG. 30 shows that the addition of 4 nucleotides and 13 nucleotides to the 3' and 5' end of SEQ ID NO: 7, respectively, results in an active molecule (e.g., SEQ ID NO: 19). Thus, ApTOLL aptamer (SEQ ID NO: 7) can be extended at either the 3' (up to 4 nucleotides) or 5' (up to 13 nucleotides) without loss of function.

- 40 Accordingly, in an embodiment, the functionally equivalent variant is a sequence with the addition of 1 to 15 nucleotides at the 5' end of SEQ ID NO: 7, particularly of 1 to 13 nucleotides. In another

embodiment, the functionally equivalent variant is a sequence with the addition of 1 to 15 nucleotides at the 3' end of SEQ ID NO: 7, particularly of 1 to 4 nucleotides. In another particular embodiment, the functionally equivalent variant is a sequence with the addition of 1 to 13 nucleotides at the 5' end and 1 to 4 nucleotides at the 3' end of SEQ ID NO: 7. In another particular embodiment, the functionally equivalent variant is SEQ ID NO: 19.

In another embodiment, the functionally equivalent variant is a sequence with the addition of 1 to 2 nucleotides to SEQ ID NO: 7 (such as SEQ ID NO: 15 and 17).

In some embodiments, the functionally equivalent variant includes a sequence with the deletion of: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides, particularly at the 3' and/or 5' ends of the sequence SEQ ID NO: 7, particularly between 1 and 15 nucleotides. Deletions at the 3' and/or 5' end of sequence SEQ ID NO: 7 are understood as fragments of SEQ ID NO 7.

In an embodiment, the functionally equivalent variant is a sequence with the deletion of 1 to 15 nucleotides at the 5' end of SEQ ID NO: 7, particularly of 1 to 6 nucleotides. In another embodiment, the functionally equivalent variant is a sequence with the deletion of 1 to 15 nucleotides at the 3' end of SEQ ID NO: 7, particularly of 1 to 11 nucleotides. In an embodiment, the functional equivalent variant is SEQ ID NO: 8.

In another embodiment, the functionally equivalent variant is a sequence with the deletion of 1 to 5 nucleotides to SEQ ID NO: 7, particularly of 1 nucleotide (such as SEQ ID NO: 16).

In some embodiments, the functionally equivalent variant is a sequence with the substitution of: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides, particularly of 1, 2, 3, 4, 5, 6, 7 and 8 nucleotides, and more particularly of 4, 5 or 6 nucleotides to SEQ ID NO: 7. Examples of functionally equivalent variants with nucleotide substitutions are SEQ ID NO: 13-18.

A person skilled in the art would understand that the number of substitutions corresponding to a certain percentage of identity to a sequence with the length of SEQ ID NO: 7, i.e., 59 nucleotides, can be easily calculated. Thus, the percentage of sequence identity can be expressed in terms of number of substitutions. For example, "at least 90% identical to SEQ ID NO: 7" could be replaced with "1 to 5 substitutions with respect to SEQ ID NO: 7".

In some embodiments, the functionally equivalent variant is a sequence with at least one addition, deletion, or substitution in the stem, the connector and/or the loop location of the secondary structure of SEQ ID NO: 7 (see e.g., FIG. 29 and FIG. 30). FIG. 29 shows the predicted secondary structure of SEQ ID NO: 7 which consists of a main loop in the center, herein referred to the "connector", different loops linked to the connector, and stems that connect the loops to the connector. The addition, deletion or substitution can be located at the stems (FIG. 29(A)), connector (FIG. 29(B)) or loops

(FIG. 29(C)). In an embodiment, the functionally equivalent variant is a sequence with at least one conservative addition, deletion, or substitution in the stem, the connector and/or the loop location.

5 EXAMPLE 9 and FIG. 29(A) show that the substitution in the stems of the loops can be effected as long as they are compensated, and that complementarity in the stem is preserved (e.g., SEQ ID NO: 13-18). A substitution in a stem implies the substitution in four nucleotides, i.e., two complementary double nucleotide substitutions. Accordingly, in some embodiments, the functionally equivalent variant is a sequence with at least two complementary double nucleotide substitutions at a stem location with respect to SEQ ID NO: 7, i.e., each stem substitution is a pair of conservative substitutions, and
10 each mutated pair is accompanied by a compensatory substitution on the complementary stem sequence. In an embodiment, the functionally equivalent variant has a substitution only at one of the stems, i.e., no substitutions at more than one stem. In some embodiments, the stem substitutions can be combined with 3' and/or 5' extensions (e.g., SEQ ID NO: 15 or SEQ ID NO: 17).

15 EXAMPLE 9 and FIG. 29(B) show that a single substitution within a connector between loops is well tolerated (e.g., SEQ ID NO: 13, 15-18). Accordingly, in some embodiments, the functionally equivalent variant is a sequence with at least one nucleotide addition, deletion or substitution at the connector. In some embodiments, the connector addition, deletion or substitutions can be combined with 5' and/or 3' extensions and stem addition, deletion or substitutions.

20 EXAMPLE 9 and FIG. 29(C) show that a single substitution or deletion within a loop is well tolerated (e.g., SEQ ID NO: 14, 16, 18). Accordingly, in some embodiments, the functionally equivalent variant is a sequence with at least one addition, deletion or substitution at a loop. In some embodiments, the loop addition, deletion or substitutions can be combined with 5' and/or 3' extensions and stem addition, deletion or substitutions.
25

In some embodiments, the connector additions, deletions or substitutions and loop additions, deletions or substitutions can be present in the same sequence (e.g., SEQ ID NO: 16 or SEQ ID NO: 18).

30 FIG. 29 shows some mutations that can be made in ApTOLL sequence, resulting in SEQ ID NO: 13-18. From these sequences, the pattern SEQ ID NO: 22 can be obtained. Accordingly, in some embodiments, the functionally equivalent variant is a sequence with the pattern **NNTGTGCCAATAAAN-NATANCGCCGCGTTAGCATGTACNNGGTTGGCCCCNAAATACNNG** (SEQ ID NO: 22), wherein **N** is any nucleotide. In another embodiment, the functionally equivalent variant is a sequence with the pattern **SSTGTGCCAATAAASSATAYCGCCGCGTTAGCATGTACHBGGTTGGCCCHAAATACVDG** (SEQ ID NO: 23), wherein S is C or G; Y is C or T; H is A or C or T; B is C or G or T; V is C or G or A; and D is A or G or T. In some embodiments, SEQ ID NO: 22 and/or 23 can be extended at 5' and/or 3' ends.
40

In some embodiments, the functionally equivalent variant is a sequence with a GC content equivalent to SEQ ID NO: 7. Accordingly, in an embodiment, the functional equivalent variant is a sequence with G or C nucleotides at positions 1, 2, 4, 6-8, 15, 16, 21-26, 31, 32, 35, 38, 40, 41, 42, 45-49, 56, 57 and 59 of SEQ ID NO: 7.

5

In some embodiments, the functionally equivalent variant has a length between: 40 and 100 nucleotides, 40 and 80 nucleotides, 40 and 70 nucleotides, 40 and 65 nucleotides, and particularly between 40 and 61 nucleotides, or between 41 and 61 nucleotides. In specific embodiments, the functionally equivalent variant has a length of: 41 nucleotides (e.g., SEQ ID NO: 8), 58 nucleotides (e.g., SEQ ID NO: 16), 59 nucleotides (e.g., SEQ ID NO: 13, 14 and 18), 60 nucleotides (e.g., SEQ ID NO: 15), or 61 nucleotides (SEQ ID NO: 17). In another particular embodiment, the functionally equivalent variant has a length between 59 and 61 nucleotides.

10

In some embodiment, the functionally equivalent variant is a sequence with respect to SEQ ID NO: 7, comprising:

15

(a) the addition, deletion, and/or substitution of 1 to 20 nucleotides at any nucleotide of the sequence;
(b) the substitution of 1 to 15 nucleotides at any nucleotide of the sequence, particularly of 1 to 6 nucleotides;

(c) the addition of 1 to 20 nucleotides at the 5' end of the sequence, particularly of 1 to 13 nucleotides;

20

(d) the addition of 1 to 20 nucleotides at the 3' end of the sequence, particularly of 1 to 4 nucleotides;

(e) the deletion of 1 to 20 nucleotides at the 5' end of the sequence, particularly of 1 to 6 nucleotides;

(f) the deletion of 1 to 20 nucleotides at the 3' end of the sequence, particularly of 1 to 11 nucleotides;

(g) at least two complementary double nucleotide substitution at a stem location of the secondary structure of the sequence;

25

(h) at least one nucleotide substitution at a connector location of the secondary structure of the sequence;

(i) at least one nucleotide substitution at a loop location of the secondary structure of the sequence;
or

(j) a combination thereof,

30

wherein the functionally equivalent variant:

- has a length between 40 and 100 nucleotides, particularly between 40 and 65 nucleotides, and more particularly between 41 and 61 nucleotides;

- has the capability of specifically binding to TLR-4 and inhibiting TLR-4 activation; and

- has the secondary structure of SEQ ID NO: 7 as determined using mFold.

35

In some embodiments, the present invention includes functionally equivalent variants which comprise a polynucleotide with a sequence identity of at least: 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% with the sequences SEQ ID NO: 1-6, and maintain the capability of specifically binding to TLR-4 and inhibiting TLR-4 activation.

40

In an embodiment, the aptamer comprises:

- (a) the polynucleotide SEQ ID NO: 7 or a fragment thereof, comprising at least 10 chemically modified nucleotides which are sugar modified nucleotides, wherein the aptamer has a length between 40 and 65 nucleotides and has the capability of specifically binding to TLR-4 and inhibiting TLR-4 activation, and wherein the fragment consists of SEQ ID NO: 8; or
- (b) a functionally equivalent variant having at least 70% sequence identity to the aptamer of (a), wherein the functionally equivalent variant maintains the capability of specifically binding to TLR-4 and inhibiting TLR-4 activation.

10 Chemical modifications

In some embodiments, the aptamer of the present invention (e.g., SEQ ID NO: 1-6) comprises at least 10 chemically modified nucleotides. Since the aptamers of the present invention are chemically modified, they can also be referred herein to as "modified aptamers" or "chemically modified aptamers".

As used herein in reference to a polynucleotide, the terms "chemical modification" or, as appropriate, "chemically modified", refer to a modification with respect to adenosine (A), guanosine (G), uridine (U), thymidine (T) or cytidine (C) ribo- or deoxyribonucleotides in one or more of their position, pattern, percent or population, including, but not limited to, its nucleobase, sugar, backbone, or any combination thereof.

Accordingly, in an embodiment, the aptamer of the present invention comprises at least 10 chemically modified nucleotides, wherein each chemically modified nucleotide is independently selected from the group consisting of: a sugar modified nucleotide (i.e., a nucleoside with a sugar modification, which is a nucleoside analog), a base modified nucleotide (i.e., nucleobase modification), a backbone modified nucleotide, and a reverse nucleoside derivative (i.e., inverted nucleoside). Particularly, the chemically modified nucleotide is a sugar modified nucleotide.

The aptamers of the present invention are provided in the form of a single-stranded DNA or RNA molecule. In an embodiment, the aptamers are DNA aptamers, of which some of the natural nucleosides/nucleotides are replaced by modified nucleosides/nucleotides. These modified nucleosides/nucleotides are similar to the natural nucleosides/nucleotides but contain extra functional groups that make the nucleases unable to digest them, and can be either deoxyribose-derived or ribose-derived. Therefore, whatever the structure of the nucleosides/nucleotides, the aptamers of the present invention are DNA aptamers comprising various types of modifications that can be 2'-deoxy ribose derivatives or ribose derivatives.

As known in the art, ribose-type nucleosides have ribose as carbohydrate with a 5-membered hemiacetal ring and three alcohol groups: 5'-OH, 2'-OH and 3'-OH. If any of the three alcohols are

missing, they are referred to as deoxyribonucleosides. For example, if the OH is missing in the position 2', they are referred to as 2'-deoxynucleosides.

Therefore, in the present invention, a nucleotide being unmodified means that there is an hydrogen
5 at the 2' position of a deoxyribonucleotide and is not substituted by any other element, while a nucleotide being modified means that the nucleotide can be either deoxyribose-derived or ribose-derived. For example, 2'-fluoro-2'-deoxy derivatives are 2'-deoxynucleosides, while 2'-O-methyl-RNA or 2'-O-MOE-RNA derivatives are ribonucleotides.

10 In some embodiments, the modified aptamer can exhibit one or more desirable properties, e.g., improved thermal or chemical stability, reduced degradation, a new therapeutic effect (e.g., against breast cancer or inflammatory autoimmune neuropathy), similar or increased binding to the TLR-4 target epitope, reduced non-specific binding to other molecules, e.g., other Toll-like receptors, as compared to the corresponding unmodified aptamer (e.g., SEQ ID NO: 7-8). In a particular embodiment,
15 the modified aptamers of the present invention exhibit a new therapeutic effect (e.g., against breast cancer or inflammatory autoimmune neuropathy) and improved stability, together with a similar binding to the TLR-4 target epitope.

Modified nucleotide base pairing encompasses not only the standard adenine-thymine, adenine-uracil,
20 or guanine-cytosine base pairs, but also base pairs formed between nucleotides and/or modified nucleotides comprising non-standard or modified bases, wherein the arrangement of hydrogen bond donors and hydrogen bond acceptors permits hydrogen bonding between a non-standard base and a standard base or between two complementary non-standard base structures. One example of such non-standard base pairing is the base pairing between the modified nucleobase inosine and adenine,
25 cytosine or uracil. Any combination of base/sugar or linker can be incorporated into an aptamer of the present invention.

1. Sugar modifications

30 The modified nucleosides and nucleotides which can be included into an aptamer of the present invention, can be modified on the sugar of the nucleic acid. Thus, in some embodiments, the aptamer of the present invention comprises at least one nucleoside analog (e.g., a nucleoside with a sugar modification). In a particular embodiment, the chemical modification is a sugar modification (e.g., SEQ ID NO: 1-6).

35 Generally, RNA includes the sugar group ribose, which is a 5-membered ring having an oxygen. Exemplary, in some embodiments, modified nucleotides include replacement of the oxygen in ribose (e.g., with sulphur (S), selenium (Se), or alkylene, such as methylene or ethylene); addition of a double bond (e.g., to replace ribose with cyclopentenyl or cyclohexenyl); ring contraction of ribose
40 (e.g., to form a 4-membered ring of cyclobutane or oxetane); ring expansion of ribose (e.g., to form

a 6- or 7-membered ring having an additional carbon or heteroatom, such as for anhydrohexitol, altritol, mannitol, cyclohexanyl, cyclohexenyl, and morpholino that also has a phosphoramidate backbone); multicyclic forms (e.g., tricyclo; and "unlocked" forms, such as glycol nucleic acid (GNA) (e.g., (*R*)-GNA or (*S*)-GNA, where ribose is replaced by glycol units attached to phosphodiester bonds),
5 threose nucleic acid (TNA, where ribose is replaced with α -L-threofuranosyl-(3'→2')), and peptide nucleic acid (PNA, where 2-amino-ethyl-glycine linkages replace the ribose and phosphodiester backbone). The sugar group can also contain one or more carbons that possess the opposite stereochemical configuration than that of the corresponding carbon in ribose. Thus, a polynucleotide molecule can include nucleotides containing, e.g., arabinose, as the sugar group.

10

In some embodiments, the nucleoside analog is a locked nucleic acid (LNA), which refers to a modified RNA nucleotide the ribose moiety of which is modified with an additional bond connecting the oxygen at 2' with the carbon at 4', locking the ribose in the 3'-endo conformation. LNA can alternatively be referred to as 2'-O, 4'-C-methylene- β -D-ribofuranosyl nucleosides (e.g., 2'-O, 4'-C-methylene- β -D-ribofuranosylthymine (LNA-T) and 2'-O, 4'-C-methylene- β -D-ribofuranosylcytosine (LNA-C)).

In some embodiments, the nucleoside analog is a peptide nucleic acid (PNA), which refers to an oligonucleotide the backbone of which is made up of repetitive units of N-(2-aminoethyl)-glycine bound by peptide bonds, wherein the different nitrogenous bases are bound to the main chain by a methylene bond (-CH₂-) and a carbonyl group -(C=O)-.

The 2' hydroxyl group (OH) of ribose can be modified or replaced with a number of different substituents. In some embodiments, exemplary substitutions at the 2'-position include, but are not limited to, H, halo, optionally substituted C₁₋₆ alkyl; optionally substituted C₁₋₆ alkoxy; optionally substituted C₆₋₁₀ aryloxy; optionally substituted C₃₋₈ cycloalkyl; optionally substituted C₃₋₈ cycloalkoxy; optionally substituted C₆₋₁₀ aryloxy; optionally substituted C₆₋₁₀ aryl-C₁₋₆ alkoxy, optionally substituted C₁₋₁₂ (heterocycl)oxy; a sugar (e.g., ribose, pentose, or any described herein); a polyethyleneglycol (PEG), -O(CH₂CH₂O)_nCH₂CH₂OR, where R is H or optionally substituted alkyl, and n is an integer from 0 to
20 (e.g., from 0 to 4, from 0 to 8, from 0 to 10, from 0 to 16, from 1 to 4, from 1 to 8, from 1 to 10, from 1 to 16, from 1 to 20, from 2 to 4, from 2 to 8, from 2 to 10, from 2 to 16, from 2 to 20, from 4 to 8, from 4 to 10, from 4 to 16, and from 4 to 20); "locked" nucleic acids (LNA) in which the 2'-hydroxyl is connected by a C₁₋₆ alkylene or C₁₋₆ heteroalkylene bridge to the 4'-carbon of the same ribose sugar, where exemplary bridges include methylene, propylene, ether, amino bridges, aminoalkyl, aminoalkoxy, amino, and amino acid.

In some embodiments, nucleoside analogues present in an aptamer of the present invention (e.g., SEQ ID NO: 1-6) comprise, e.g., 2'-O-alkyl-RNA units, 2'-OMe-RNA units, 2'-O-alkyl-RNA, 2'-amino-DNA units, 2'-fluoro-DNA units, LNA units, arabinonucleic acid (ANA) units, 2'-fluoro-ANA units, hexitol nucleic acid (HNA) units, intercalating nucleic acid (INA) units, 2'-MOE units, or any

40

combination thereof. In some embodiments, the LNA is, e.g., oxy-LNA (such as beta-D-oxy-LNA, or alpha-L-oxy-LNA), amino-LNA (such as beta-D-amino-LNA or alpha-L-amino-LNA), thio-LNA (such as beta-D-thio-LNA or alpha-L-thio-LNA), ENA (such a beta-D-ENA or alpha-L-ENA), or any combination thereof.

5

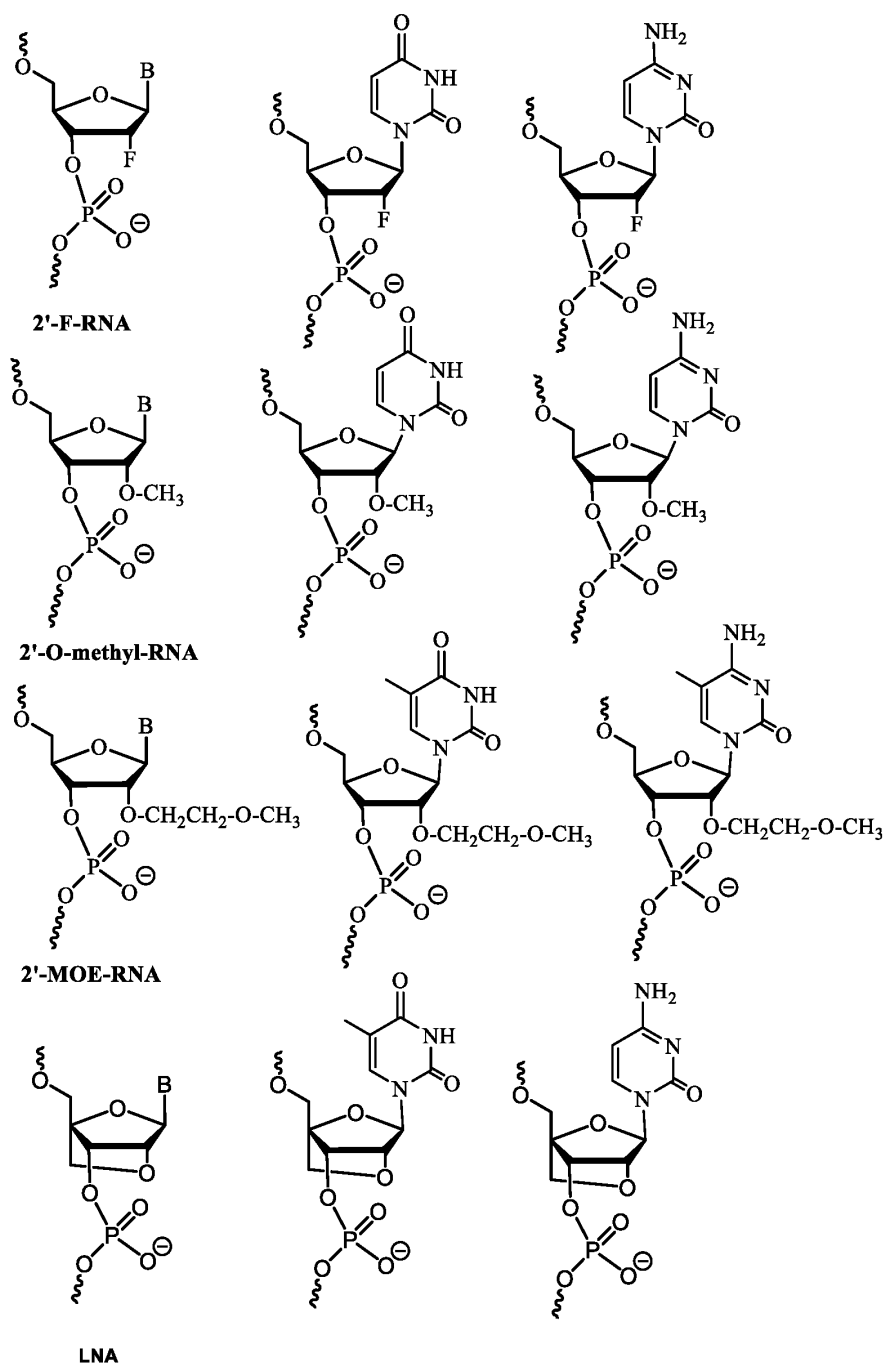
In some embodiments, nucleoside analogs present in an aptamer of the present invention (e.g., SEQ ID NO: 1-6) comprise LNA; 2'-O-alkyl-RNA; 2'-amino-DNA; 2'-fluoro-DNA; ANA; 2'-fluoro-ANA, HNA, INA, constrained ethyl nucleotide (cEt), 2'-O-methyl nucleic acid (2'-OMe), 2'-O-methoxyethyl nucleic acid (2'-MOE), or any combination thereof.

10

In some embodiments, an aptamer of the present invention (e.g., SEQ ID NO: 1-6) can comprise both modified RNA nucleotide analogues (e.g., LNA) and DNA units. See, e.g., U.S. Pat. Nos. 8,404,649; 8,580,756; 8,163,708; and 9,034,837; all of which are herein incorporated by reference in their entireties.

15

In some embodiments, the aptamer of the present invention comprises at least one sugar modification as disclosed below.



In some embodiments, the sugar modification includes a nucleoside analog (modification or analog form of ribose or deoxyribose) including without limitation sugars substituted at 2', such as 2'-F-RNA or 2'-F-2'-deoxynucleosides (e.g., 2'-F-2'-deoxyuridine (2'-F-2'-dU) and 2'-F-2'-deoxycytidine (2'-F-2'-dC)), 2'-O-methyl-RNA or 2'-O-methylnucleosides (e.g., 2'-O-methyl-uridine (2'-OMe-U) and 2'-O-methylcytidine (2'-OMe-C)), 2'-O-methoxyethyl-RNA or 2'-O-MOE-nucleosides (e.g., 2'-O-

methoxyethyl-5-methyluridine (2'-O-MOE-T) and 2'-O-methoxyethyl-5-methylcytidine (2'-O-MOE-meC)), or 2'-azido-ribose, carbocyclic analogues of sugars, α -anomeric sugars, epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars and sedoheptuloses. In particular, the substitution in the position 2' of the residue of furanose is particularly important with respect to the improvement in nuclease stability.

The aptamers can also comprise threose nucleic acid (TNA, also referred to as α -threofuranosyl oligonucleotides).

In an embodiment, the sugar modification is a nucleoside analog such as LNA or 2'-substituted sugars. In a particular embodiment, the nucleoside analog is a LNA. In another embodiment, the nucleoside analog is a 2'-substituted sugar.

In a particular embodiment, each sugar modified nucleotide is independently selected from the group consisting of: 2'-fluoro-2'-deoxynucleoside (2'-F-RNA), 2'-O-methoxyethyl-nucleoside (2'-O-MOE-RNA), 2'-O-methylnucleoside (2'-O-methyl-RNA), and 2'-O, 4'-C-methylene- β -D-ribofuranosyl nucleoside (LNA or locked nucleic acid).

Particularly, each sugar modification is independently selected from the group consisting of: 2'-fluoro-2'-deoxyuridine (2'-F-2'-dU), 2'-fluoro-2'-deoxycytidine (2'-F-2'-dC), 2'-O-methoxyethyl-5-methyluridine (2'-O-MOE-T), 2'-O-methoxyethyl-5-methylcytidine (2'-O-MOE-meC), 2'-O-methyluridine (2'-OMe-U), 2'-O-methylcytidine (2'-OMe-C), 2'-O, 4'-C-methylene- β -D-ribofuranosylthymine (LNA-T), and 2'-O, 4'-C-methylene- β -D-ribofuranosylcytosine (LNA-C).

2. Base modifications

In some embodiments, the chemical modification is at nucleobases in an aptamer of the present invention. In some embodiments, the chemically modified nucleoside is a modified uridine (e.g., pseudouridine (ψ), 2-thiouridine (s2U), 1-methyl-pseudouridine (m1 ψ), 1-ethyl-pseudouridine (e1 ψ), or 5-methoxy-uridine (mo5U)), a modified cytosine (e.g., 5-methyl-cytidine (m5C)) a modified adenosine (e.g., 1-methyl-adenosine (m1A), N6-methyl-adenosine (m6A), or 2-methyl-adenine (m2A)), or a modified guanosine (e.g., 7-methyl-guanosine (m7G) or 1-methyl-guanosine (m1G)).

In some embodiments, the aptamer of the present invention comprises analogue forms of purines and pyrimidines which include, without limitation, aziridinylcytosine, 4-acetylcytosine, 5-fluorouracil, 5-bromouracil, 5-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyluracil, inosine, N⁶-isopentenyladenine, 1-methyladenine, 1-methylpseudouracil, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N⁶-methyladenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylkeosine, 5-methoxyuracil, 2-methylthio-N-6-isopentenyladenine, uracil-

5-oxyacetic acid methyl ester, pseudouracil, keosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid, and 2,6-diaminopurine. In addition to the preceding modified nucleotides, nucleotide residues lacking a purine or a pyrimidine also can be included in the aptamer of the present invention.

5

3. Backbone modifications

In some embodiments, the aptamer of the present invention comprises a modification to the linkages between the nucleosides. Non-limiting backbone modifications include: 3'-alkylene phosphonates, 3'-amino phosphoramidate, alkene containing backbones, aminoalkylphosphoramidates, aminoalkylphosphotriesters, boranophosphates, $-\text{CH}_2-\text{O}-\text{N}(\text{CH}_3)-\text{CH}_2-$, $-\text{CH}_2-\text{N}(\text{CH}_3)-\text{N}(\text{CH}_3)-\text{CH}_2-$, $-\text{CH}_2-\text{NH}-\text{CH}_2-$, chiral phosphonates, chiral phosphorothioates, formacetyl and thioformacetyl backbones, methylene (methylimino), methylene formacetyl and thioformacetyl backbones, methyleneimino and methylenehydrazino backbones, morpholino linkages, $-\text{N}(\text{CH}_3)-\text{CH}_2-\text{CH}_2-$, oligonucleosides with heteroatom internucleoside linkage, phosphinates, phosphoramidates, phosphorodithioates, phosphorothioate internucleoside linkages, phosphorothioates, phosphotriesters, PNA, LNA siloxane backbones, sulfamate backbones, sulfide sulfoxide and sulfone backbones, sulfonate and sulfonamide backbones, thionoalkylphosphonates, thionoalkylphosphotriesters, and thionophosphoramidates.

20

In some embodiments, the presence of a backbone linkage increases the stability (e.g., thermal stability) and/or resistance to degradation (e.g., enzyme degradation) of an aptamer of the present invention.

25 In some embodiments, the chemical modification includes structures with analogue synthetic backbones of the typical phosphodiester backbone. These include, without limitation, phosphorothioate, phosphorodithioate, methylphosphonate, phosphoramidate, phosphodiester, phosphotriester, sulfamate, 3'-thioacetal, methylene(methylimino), 3'-N-carbamate, or morpholine carbamate.

30 Alternative binding groups include, in a non-limiting manner, embodiments in which a moiety of formula $\text{P}(\text{O})\text{S}$, ("thioate"), $\text{P}(\text{S})\text{S}$ ("dithioate"), $\text{P}(\text{O})\text{NR}'_2$, $\text{P}(\text{O})\text{R}'$, $\text{P}(\text{O})\text{OR}^6$, CO , or CONR'_2 , wherein R' is H (or a salt) or an alkyl group of 1-12 carbon atoms and R^6 is an alkyl group of 1-9 carbon atoms, which binds to adjacent nucleotides through -S- or -O-. The present invention also contemplates the use of substitution bonds including internucleotide bonds not based on phosphorus, such as 3'-thioformacetal, (-S-CH₂-O-), formacetal (-O-CH₂-O-) and 3'-amine internucleotide bonds (-NH-CH₂-CH₂-).

40 Not all the bonds within the same aptamer have to be identical, and the present invention therefore contemplates aptamers with all identical bonds as well as aptamers with a variation in the composition of their bonds.

In some embodiments, the chemical modification includes a chemical substitution at the phosphate moiety, e.g., phosphorothioation, N-mesylphosphoramidation, amino group, chemical modification of lower alkylamine groups, and acetyl groups, among others.

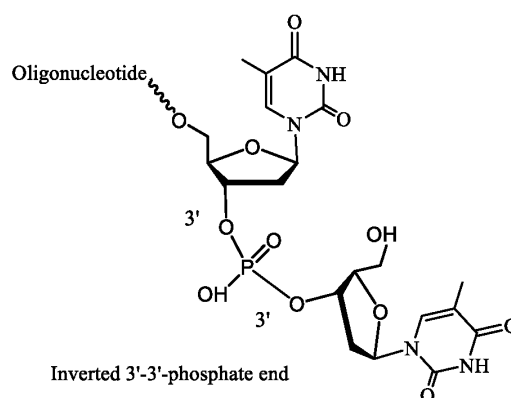
5

4. Reverse nucleoside derivatives

In standard oligonucleotide synthesis, the assembly of the oligonucleotides starts from the 3'-end towards the 5'-end. The first nucleoside is attached in a solid support through the 3'-position carrying a dimethoxytrityl (DMT) group at the 5'-position, which is a 5' protecting group. The rest of the nucleotides are added in the form of 5'-DMT-protected 3'-phosphoramidite.

In the reverse nucleoside derivatives, the DMT group is attached at the 3'-position and the phosphoramidite or solid support is attached to the 5'-end. The introduction of the inverted 3'-end is made by using a solid support functionalized with the reverse nucleoside with the DMT group at the 3'-position. When removing the DMT group at the 3'-position of the first nucleoside, the addition of a standard 5'-DMT-protected 3'-phosphoramidite generates a 3'-3' phosphate bond that is not recognized by exonucleases avoiding the degradation of the oligonucleotide by exonucleases (see figure below).

15



20

5'-5' chain or 3'-3' chain refers herein to oligonucleotides in which the nucleotide of the 3' or 5' ends, respectively, is inverted.

In an embodiment, the aptamer of the present invention comprises a reverse nucleoside derivative. In a particular embodiment, the reverse nucleoside derivative (inverted nucleoside) is thymidinyl (3'-3') phosphate thymidine (inverted T), or 2'-deoxycytidinyl (3'-3') phosphate 2'-deoxycytidine (inverted C).

25

30 Addition of inverted nucleotides

As explained, the aptamer of the present invention can optionally comprise the addition of at least one inverted nucleotide at the 5' and/or 3' ends with respect to the reference sequence (e.g., SEQ ID NO: 7-8 and variants thereof). In a particular embodiment, the aptamer comprises additional inverted nucleotides at the 3' end.

5

In a particular embodiment, the aptamer comprises between 1 and 5 additional inverted nucleotides. The additional nucleotide can be an inverted nucleoside of the 3' or 5' end, thereby forming an oligonucleotide with a 5'-5' chain or 3'-3' chain. Particularly, the additional inverted nucleotides are at the 3' end.

10

In a particular embodiment, the additional nucleotide is an inverted nucleoside, particularly thymidinyl (3'-3') phosphate thymidine (inverted T), or 2'-deoxycytidinyl (3'-3') phosphate 2'-deoxycytidine (inverted C).

15 In a particular embodiment, the aptamer comprises between 1 and 5 additional nucleotides at the 3' end, wherein the additional nucleotide is an inverted nucleoside, particularly inverted T, or inverted C.

20 In a particular embodiment, the aptamer comprises 1 additional nucleotide at the 3' end, wherein the additional nucleotide is an inverted T (e.g., position 60 of ApSION 1-5, SEQ ID NO: 1-5). This inverted T would correspond to the addition of 1 inverted nucleotide to position 60 of SEQ ID NO: 7.

25 In a particular embodiment, the aptamer comprises 1 additional nucleotide at the 3' end, wherein the additional nucleotide is an inverted C (e.g., position 42 of ApSION 6, SEQ ID NO: 6). This inverted C would correspond to the addition of 1 inverted nucleotide to position 42 of SEQ ID NO: 8, or alternatively to position 48 of SEQ ID NO: 7.

Number and position of the chemical modifications

30 The modified aptamers of the present invention can comprise various distinct modifications. In some embodiments, the modified aptamer contains one or more (optionally different) nucleoside/nucleotide modifications.

35 In some embodiments, the aptamers of the present invention (e.g., SEQ ID NO: 1-6) can have a uniform chemical modification of all or any of the same nucleoside type or a population of modifications in all or any of the same nucleoside type, or a measured percent of a chemical modification of all any of the same nucleoside type but with random incorporation.

40 In a particular embodiment, the aptamers of the present invention (e.g., SEQ ID NO: 1-6) can have a uniform chemical modification of two, three, or four of the same nucleoside type throughout the

entire polynucleotide (such as all thymidines and/or all cytidines, etc. are modified in the same way). In a particular embodiment, the chemically modified nucleoside/nucleotide is a nucleotide with a pyrimidine nitrogenous base (i.e., thymine or T, cytosine or C, and uracil or U).

5 In some embodiments, the aptamer of the present invention (e.g., SEQ ID NO: 1-6) is uniformly modified (e.g., partially or fully modified, modified throughout the entire sequence) for a particular modification or for a group of modifications. For example, an aptamer can comprise both 2'-fluoro-2'-deoxynucleosides and LNAs in some of its residues, or can only comprise 2'-fluoro-2'-deoxynucleosides in some of its residues. In a particular embodiment, the aptamer comprises the same type
10 of chemical modification throughout the entire aptamer.

In some embodiments, the nucleobases, sugar groups, backbone linkages, or any combination thereof in an aptamer of the present invention (e.g., SEQ ID NO: 1-6) are modified by at least about: 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%,
15 95%, or 100%. In a particular embodiment, the aptamer is modified by between about 25% and 55%.

In an embodiment, at least about: 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% of the nucleotides in an aptamer of the present invention (e.g., SEQ ID NO: 1-6) contain sugar modifications (e.g., LNA) or inverted
20 nucleotides. In a particular embodiment, the aptamer comprises sugar modifications or inverted nucleotides by between about 25% and 55%.

In some embodiments, the aptamer of the present invention (e.g., SEQ ID NO: 1-6) comprises at least: 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50,
25 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 chemically modified nucleotides (e.g., sugar modified nucleotides or inverted nucleotides). In a particular embodiment, the aptamer comprises at least 16, at least 23, or at least 29 chemically modified nucleotides.

In some embodiments, the aptamer of the present invention (e.g., SEQ ID NO: 1-6) comprises between 10 and 30 chemically modified nucleotides, between 15 and 30 chemically modified nucleotides, and particularly between 16 and 29 chemically modified nucleotides. In an embodiment, the
30 aptamer of the present invention (e.g., SEQ ID NO: 1-6) comprises 16, 23, or 29 chemically modified nucleotides.

35 In some embodiments, the aptamer of the present invention (e.g., SEQ ID NO: 1-6) comprises at least 10 chemically modified T or C nucleotides. In other embodiments, the aptamer of the present invention (e.g., SEQ ID NO: 1-6) comprises at least 16 chemically modified T or C nucleotides. In other embodiments, the aptamer of the present invention (e.g., SEQ ID NO: 1-6) comprises at least 23 chemically modified T or C nucleotides. In other embodiments, the aptamer of the present inven-
40 tion (e.g., SEQ ID NO: 1-6) comprises at least 29 chemically modified T or C nucleotides. Particularly,

each chemically modified T and/or C nucleotide is independently selected from the group consisting of: 2'-fluoro-2'-deoxyuridine (2'-F-2'-dU), 2'-fluoro-2'-deoxycytidine (2'-F-2'-dC), 2'-O-methoxyethyl-5-methyluridine (2'-O-MOE-T), 2'-O-methoxyethyl-5-methylcytosine (2'-O-MOE-meC), LNA-T, LNA-C, 2'-O-methyluridine (2'-OMe-U), 2'-O-methylcytidine (2'-OMe-C), inverted T, and inverted C.

5

In some embodiments, the aptamer of the present invention (e.g., SEQ ID NO: 1-6) comprises: i) at least 9 chemically modified T nucleotides, and ii) at least 7 chemically modified C nucleotides. In other embodiments, the aptamer (e.g., SEQ ID NO: 1-4 and 6) comprises i) at least 10 chemically modified T nucleotides, and ii) at least 13 chemically modified C nucleotides. In other embodiments, the aptamer (e.g., SEQ ID NO: 1-4) comprises: i) at least 14 chemically modified T nucleotides, and ii) at least 15 chemically modified C nucleotides. Particularly, each chemically modified T nucleotide is independently selected from the group consisting of: 2'-fluoro-2'-deoxyuridine (2'-F-2'-dU), 2'-O-methoxyethyl-5-methyluridine (2'-O-MOE-T), 2'-O-methyluridine (2'-OMe-U), LNA-T, and inverted T; and each chemically modified C nucleotide is independently selected from the group consisting of: 2'-fluoro-2'-deoxycytidine (2'-F-2'-dC), 2'-O-methoxyethyl-5-methylcytosine (2'-O-MOE-meC), 2'-O-methylcytidine (2'-OMe-C), LNA-C and inverted C.

In some embodiments, the aptamer of the present invention (e.g., SEQ ID NO: 1-6) comprises at least one nucleotide addition at the 3' end. In an embodiment, the aptamer (e.g., SEQ ID NO: 1-6) comprises an additional nucleotide at the 3' end. Particularly, the additional nucleotide is an inverted nucleotide, particularly an inverted T or an inverted C.

The chemical modifications can be located in any of the nucleotides of the aptamer of the present invention or in specific positions, such as in residues 3, 5, 7, 8, 11, 15, 16, 18, 20, 21, 23, 24, 26, 28, 29, 32, 34, 36, 38, 39, 40, 43, 44, 47, 48, 49, 50, 54 and 56 of the sequence (e.g., SEQ ID NO: 1-6).

In some embodiments, the chemically modified nucleotide is located at any of residues 3, 5, 7, 8, 11, 15, 16, 18, 20, 21, 23, 24, 26, 28, 29, 32, 34, 36, 38, 39, 40, 43, 44, 47, 48, 49, 50, 54 and 56 of the reference sequence SEQ ID NO: 7.

30

In a particular embodiment, the chemically modified nucleotide is located at residues 7, 8, 11, 18, 20, 23, 24, 28, 29, 34, 38, 39, 40, 43, 44, and 47 of SEQ ID NO: 7 (e.g., ApSION 1-6, SEQ ID NO: 1-6); or the chemically modified nucleotide is located at residues 7, 8, 11, 15, 16, 18, 20, 21, 23, 24, 26, 28, 29, 32, 34, 36, 38, 39, 40, 43, 44, 47, and 48 of SEQ ID NO: 7 (e.g., ApSION 1-4 and 6, SEQ ID NO: 1-6 and 6); or the chemically modified nucleotide is located at residues 3, 5, 7, 8, 11, 15, 16, 18, 20, 21, 23, 24, 26, 28, 29, 32, 34, 36, 38, 39, 40, 43, 44, 47, 48, 49, 50, 54 and 56 of SEQ ID NO: 7 (e.g., ApSION 1-4, SEQ ID NO: 1-4).

35

In some embodiments, the chemically modified T nucleotides are located at residues 11, 18, 20, 28, 29, 34, 39, 43 and 44 of SEQ ID NO: 7; and the chemically modified C nucleotides are located at residues 7, 8, 23, 24, 38, 40 and 47 of SEQ ID NO: 7 (e.g., ApSION 1-6, SEQ ID NO: 1-6).

5 In other embodiments, the chemically modified T nucleotides are located at residues 11, 18, 20, 28, 29, 34, 36, 39, 43 and 44 of SEQ ID NO: 7; and the chemically modified C nucleotides are located at residues 7, 8, 15, 16, 21, 23, 24, 26, 32, 38, 40, 47 and 48 of SEQ ID NO: 7 (e.g., ApSION 1-4 and 6, SEQ ID NO: 1-4 and 6).

10 In other embodiments, the chemically modified T nucleotides are located at residues 3, 5, 11, 18, 20, 28, 29, 34, 36, 39, 43, 44, 50, and 54 of SEQ ID NO: 7; and the chemically modified C nucleotides are located at residues 7, 8, 15, 16, 21, 23, 24, 26, 32, 38, 40, 47, 48, 49, and 56 of SEQ ID NO: 7 (e.g., ApSION 1-4, SEQ ID NO: 1-4).

15 In a particular embodiment, the aptamer comprises 1 additional nucleotide at the 3' end, wherein the additional nucleotide is an inverted T (e.g., ApSION 1-5, SEQ ID NO: 1-5). This inverted T would correspond to position 60 of SEQ ID NO: 7.

20 In a particular embodiment, the chemically modified nucleotide is located at residues 1, 2, 5, 9, 10, 12, 14, 15, 17, 18, 20, 22, 23, 26, 28, 30, 32, 33, 34, 36, 37, 38, and 41 of SEQ ID NO: 8 (e.g., ApSION 6, SEQ ID NO: 6).

25 In another particular embodiment, the chemically modified T nucleotides are located at residues 5, 12, 14, 22, 23, 28, 30, 33, 37, and 38 of SEQ ID NO: 8; and the chemically modified C nucleotides are located at residues 1, 2, 9, 10, 15, 17, 18, 20, 26, 32, 34, 36, and 41 of SEQ ID NO: 8 (e.g., ApSION 6, SEQ ID NO: 6).

30 In a particular embodiment, the aptamer comprises 1 additional nucleotide at the 3' end, wherein the additional nucleotide is an inverted C (e.g., ApSION 6, SEQ ID NO: 6). This inverted C would correspond to position 42 of SEQ ID NO: 8, or alternatively to position 48 of SEQ ID NO: 7.

Particular embodiments

35 In some embodiments, each chemically modified nucleotide is independently selected from the group consisting of: 2'-substituted sugars, 2'-O, 4'-C-methylene- β -D-ribofuranosyl nucleoside (LNA or locked nucleic acid), and inverted nucleosides.

In an embodiment, each chemically modified nucleotide is independently selected from the group consisting of: 2'-fluoro-2'-deoxynucleoside (2'-F-RNA), 2'-O-methoxyethyl-nucleoside (2'-O-MOE-

RNA), 2'-O-methylnucleoside (2'-O-methyl-RNA), 2'-O, 4'-C-methylene-β-D-ribofuranosyl nucleoside (LNA or locked nucleic acid), and inverted nucleosides.

In a particular embodiment, each chemically modified nucleotide is independently selected from the group consisting of: 2'-fluoro-2'-deoxyuridine (2'-F-2'-dU), 2'-fluoro-2'-deoxycytidine (2'-F-2'-dC), 2'-O-methoxyethyl-5-methyluridine (2'-O-MOE-T), 2'-O-methoxyethyl-5-methylcytidine (2'-O-MOE-meC), 2'-O-methyluridine (2'-OMe-U), 2'-O-methylcytidine (2'-OMe-C), 2'-O, 4'-C-methylene-β-D-ribofuranosylthymine (LNA-T), 2'-O, 4'-C-methylene-β-D-ribofuranosylcytosine (LNA-C), thymidinyl (3'-3') phosphate thymidine (inverted T), and 2'-deoxycytidinyl (3'-3') phosphate 2'-deoxycytidine (inverted C).

In other embodiments, the aptamers comprise similar modifications as the above mentioned, in A and/or G nucleotides.

In a particular embodiment, the aptamer of the present invention (e.g., ApSION 1-6, SEQ ID NO: 1-6) comprises at least 10 chemically modified T and/or C nucleotides, wherein each modified T/C modified nucleotide is independently selected from the group consisting of: 2'-F-2'-dU, 2'-F-2'-dC, 2'-O-MOE-T, 2'-O-MOE-meC, 2'-OMe-U, 2'-OMe-C, 2LNA-T, LNA-C, inverted T, and inverted C.

In a particular embodiment, the aptamer of the present invention (e.g., ApSION 1-6, SEQ ID NO: 1-6) comprises:

- i) at least 9 chemically modified T nucleotides, wherein each modified T nucleotide is independently selected from the group consisting of: 2'-F-2'-dU, 2'-O-MOE-T, 2'-O-Me-U, LNA-T, and inverted T;
- ii) at least 7 chemically modified C nucleotides, wherein each modified C nucleotide is independently selected from the group consisting of: 2'-F-2'-dC, 2'-O-MOE-meC, 2'-OMe-C, LNA-C and inverted C;
- and
- iii) at least one additional nucleotide at 3' end of the sequence which is an inverted T or an inverted C.

Particularly, the chemically modified T nucleotides are located at residues 11, 18, 20, 28, 29, 34, 39, 43, and 44 of SEQ ID NO: 7, and the chemically modified C nucleotides are located at residues 7, 8, 23, 24, 38, 40, and 47 of SEQ ID NO: 7.

In a particular embodiment, the aptamer of the present invention (e.g., ApSION 1-4 and 6, SEQ ID NO: 1-4 and 6) comprises:

- i) at least 10 chemically modified T nucleotides, wherein each modified T nucleotide is independently selected from the group consisting of: 2'-F-2'-dU, 2'-O-MOE-T, 2'-O-Me-U, LNA-T, and inverted T;
- ii) at least 13 chemically modified C nucleotides, wherein each modified C nucleotide is independently selected from the group consisting of: 2'-F-2'-dC, 2'-O-MOE-meC, 2'-OMe-C, LNA-C and inverted C; and

iii) at least one additional nucleotide at 3' end of the sequence which is an inverted T or an inverted C.

Particularly, the chemically modified T nucleotides are located at residues 11, 18, 20, 28, 29, 34, 36, 39, 43, and 44 of SEQ ID NO: 7, and the chemically modified C nucleotides are located at residues 7, 8, 15, 16, 21, 23, 24, 26, 32, 38, 40, 47, and 48 of SEQ ID NO: 7.

In a particular embodiment, the aptamer of the present invention (e.g., ApSION 1-4, SEQ ID NO: 1-4) comprises:

- i) at least 14 chemically modified T nucleotides, wherein each modified T nucleotide is independently selected from the group consisting of: 2'-F-2'-dU, 2'-O-MOE-T, 2'-O-Me-U, LNA-T, and inverted T;
- ii) at least 15 chemically modified C nucleotides, wherein each modified C nucleotide is independently selected from the group consisting of: 2'-F-2'-dC, 2'-O-MOE-meC, 2'-OMe-C, LNA-C and inverted C; and
- iii) at least one additional nucleotide at 3' end of the sequence which is an inverted T or an inverted C.

Particularly, the chemically modified T nucleotides are located at residues 3, 5, 11, 18, 20, 28, 29, 34, 36, 39, 43, 44, 50 and 54 of SEQ ID NO: 7, and the chemically modified C nucleotides are located at residues 7, 8, 15, 16, 21, 23, 24, 26, 32, 38, 40, 47, 48, 49, and 56 of SEQ ID NO: 7.

In some embodiments, the aptamer of the present invention comprises the same type of chemical modification throughout the entire sequence. In an embodiment, the chemically modified nucleoside/nucleotide is a nucleotide with a pyrimidine nitrogenous base (i.e., thymine or T, cytosine or C, and uracil or U). In some embodiments, the chemically modified nucleotide is selected from the group consisting of: 2'-fluoro-2'-deoxynucleoside (2'-F-RNA), 2'-O-methoxyethyl-nucleoside (2'-O-MOE-RNA), 2'-O-methylnucleoside (2'-O-methyl-RNA), 2'-O, 4'-C-methylene- β -D-ribofuranosyl nucleoside (LNA or locked nucleic acid), and inverted nucleosides. In a particular embodiment, the chemically modified nucleoside/nucleotide is a 2'-fluoro-2'-deoxynucleoside (e.g., ApSION 1, SEQ ID NO: 1). In another particular embodiment, the chemically modified nucleoside and/or nucleotide is a 2'-O-methoxyethyl-nucleoside (e.g., ApSION 2, SEQ ID NO: 2). In another particular embodiment, the chemically modified nucleoside and/or nucleotide is a 2'-O-methylnucleoside (e.g., ApSION 4-6, SEQ ID NO: 4-6). In another particular embodiment, the chemically modified nucleoside and/or nucleotide is a 2'-O, 4'-C-methylene- β -D-ribofuranosyl nucleoside (LNA or locked nucleic acid) (e.g., ApSION 3, SEQ ID NO: 3).

In a particular embodiment, the aptamer of the present invention comprises:

at least one 2'-fluoro-2'-deoxyuridine (2'-F-2'-dU) in any of the following T residues: 3, 5, 11, 18, 20, 28, 29, 34, 36, 39, 43, 44, 50 and 54 of SEQ ID NO: 7, and at least one 2'-fluoro-2'-deoxycytidine (2'-

- F-2'-dC) in any of the following C residues: 7, 8, 15, 16, 21, 23, 24, 26, 32, 38, 40, 47, 48, 49, and 56 of SEQ ID NO: 7 (e.g., ApSION 1, SEQ ID NO: 1); or
- at least one 2'-O-methoxyethyl-5-methyluridine (2'-O-MOE-T) in any of the following T residues: 3, 5, 11, 18, 20, 28, 29, 34, 36, 39, 43, 44, 50 and 54 of SEQ ID NO: 7, and at least one 2'-O-methoxyethyl-5-methylcytosine (2'-O-MOE-meC) in any of the following C residues: 7, 8, 15, 16, 21, 23, 24, 26, 32, 38, 40, 47, 48, 49, and 56 of SEQ ID NO: 7 (e.g., ApSION 2, SEQ ID NO: 2); or
- at least one 2'-O-methyluridine (2'-OMe-U) in any of the following T residues: 3, 5, 11, 18, 20, 28, 29, 34, 36, 39, 43, 44, 50 and 54 of SEQ ID NO: 7, and at least one 2'-O-methylcytidine (2'-OMe-C) in any of the following C residues: 7, 8, 15, 16, 21, 23, 24, 26, 32, 38, 40, 47, 48, 49, and 56 of SEQ ID NO: 7 (e.g., ApSION 4-6, SEQ ID NO: 4-6); or
- at least one LNA-T in any of the following T residues: 3, 5, 11, 18, 20, 28, 29, 34, 36, 39, 43, 44, 50 and 54 of SEQ ID NO: 7, and at least one LNA-C in any of the following C residues: 7, 8, 15, 16, 21, 23, 24, 26, 32, 38, 40, 47, 48, 49, and 56 of SEQ ID NO: 7 (e.g., ApSION 3, SEQ ID NO: 3); or
- at least one inverted C in residue 42 of SEQ ID NO: 8, or alternatively in residue 48 of SEQ ID NO: 7 (e.g. ApSION 6, SEQ ID NO: 6).

- In a particular embodiment, the aptamer of the present invention comprises: at least one additional inverted T at 3' end of the sequence (i.e., residue 60 of SEQ ID NO: 7) (e.g., ApSION 1-5, SEQ ID NO: 1-5); or at least one additional inverted C at 3' end of the sequence (i.e., residue 42 of SEQ ID NO: 8, or alternatively residue 48 of SEQ ID NO: 7) (e.g., ApSION 6, SEQ ID NO: 6).

- In a particular embodiment, the aptamer of the present invention is selected from the group consisting of SEQ ID NO: 1-6 (ApSION 1-6), shown below. A, G, C, T are each a deoxyribonucleotide wherein the base is adenine, guanine, cytosine or thymine. Lowercase letters followed by the nucleotide in capital letters indicate modification of the nucleotide: fU and fC indicates that there is a fluorine atom at the 2'-position of the ribose, modification referred to as 2'-fluoro-2'-deoxyuridine (2'-F-2'-dU) and 2'-fluoro-2'-deoxycytidine (2'-F-2'-dC), respectively; eT or eC indicates that there is an O-methoxyethyl group at the 2'-position of the ribose, modification referred to as 2'-O-methoxyethyl-5-methyluridine (2'-O-MOE-T) and 2'-O-methoxyethyl-5-methylcytosine (2'-O-MOE-meC), respectively; IT and IC indicates that the nucleotide is LNA-modified, modification referred to as LNA-T and LNA-C, respectively; mU and mC indicates that there is an O-methyl group at the 2'-position of the ribose, modification referred to as 2'-O-methyluridine (2'-OMe-U) and 2'-O-methylcytidine (2'-OMe-C), respectively; and iT and iC indicates that the nucleotide is inverted, modification referred to as inverted T or inverted C, respectively.

35

ApSION 1 (SEQ ID NO: 1):

GGfU GfUG fCfCA AfUA AAfC fCAfU AfUfC GfCfC GfCG fUfUA GfCA fUGfU AfCfU fCGG fUfUG GfCfC fCfUA AAfU AfCG AGiT

- 40 ApSION 2 (SEQ ID NO: 2):

GGeT GeTG eCeCA AeTA AAeC eCAeT AeTeC GeCeC GeCG eTeTA GeCA eTGeT AeCeT eCGG
eTeTG GeCeC eCeTA AAeT AeCG AGiT

ApSION 3 (SEQ ID NO: 3):

5 GGIT GITG ICICA AITA AAIC ICAIT AITIC GICIC GICG ITITA GICA ITGIT AICIT ICGG ITITG GICIC
ICITA AAIT AICG AGiT

ApSION 4 (SEQ ID NO: 4):

GGmU GmUG mCmCA AmUA AAmC mCAmU AmUmC GmCmC GmCG mUmUA GmCA mUGmU
10 AmCmU mCGG mUmUG GmCmC mCmUA AAmU AmCG AGiT

ApSION 5 (SEQ ID NO: 5):

GGT GTG mCmCA AmUA AAC CA mU AmUC GmCmC GCG mUmUA GCA mUGT AmCmU mCGG
mUmUG GmCC CTA AAT ACG AGiT
15

ApSION 6 (SEQ ID NO: 6):

mCmCA AmUA AAmC mCAmU AmUmC GmCmC GmCG mUmUA GmCA mUGmU AmCmU
mCGG mUmUG GmCiC

20 In a particular embodiment, the aptamer of the present invention is SEQ ID NO: 2 and 4. In a particular embodiment, the aptamer is SEQ ID NO: 2 (ApSION 2). In another particular embodiment, the aptamer is SEQ ID NO: 4 (ApSION 4).

Complex comprising an aptamer

25 As the person skilled in the art will appreciate, the features of the small size, stability and easy production of the aptamer of the invention enable said aptamer to be bound to a second molecule. That is particularly advantageous when the second molecule is a functional group. The result of the binding of the aptamer of the invention and a functional group is a complex presenting the combination
30 of functions of both, i.e., a complex with the capability of specifically binding to TLR-4 and inhibiting TLR-4 and with the activity associated with the functional group.

Therefore, another aspect of the invention refers to a complex, hereinafter referred to as the "complex of the invention", comprising an aptamer as disclosed herein and a functional group.

35 The term "aptamer" has been described in detail in relation to the "Definitions" and the "Chemically modified aptamer" sections and its definitions and particularities apply likewise in the context of the complex of the invention.

The term "functional group", in the context of the present invention, refers to compounds suitable for performing at least one function. Said function includes, without limitation, the capability of binding specifically to TLR-4 or to other receptors TLR, the capability of inhibiting TLR-4 or other receptors TLR, the capability of being both directly and indirectly detectable, the capability of inducing cell death, the capability of carrying a therapeutic payload, etc. As the person skilled in the art will understand, a functional group can have associated therewith one or multiple functions. Non-limiting examples of functional groups include detectable reagents and drugs. These functional groups act like imaging agents, drugs, etc.

Therefore, in some embodiments, the functional group is selected from a detectable reagent, a drug and a nanoparticle.

In an embodiment, the functional group is a detectable reagent. The terms "detectable reagent", "imaging agent" and "contrast agent" are used herein interchangeably and refer to a biocompatible compound, the use of which facilitates the differentiation of different parts of an image, by increasing the contrast between those different regions of the image. The term "contrast agents" thus encompasses agents that are used to enhance the quality of an image that may nonetheless be generated in the absence of such an agent (as is the case, e.g., in magnetic resonance imaging-MRI), as well as agents that are prerequisites for the generation of an image (as is the case, e.g., in nuclear imaging). Suitable contrast agent include, without limitation, contrast agents for radionuclide imaging, for computerized tomography (CT), for Raman spectroscopy, for MRI, and for optical imaging.

In certain embodiments, a complex according to the invention is used for positron emission tomography (PET) or SPECT imaging *in vivo*. Detectable reagents for radionuclide imaging include radiopharmaceuticals commonly labeled with positron-emitters such as ^{11}C , ^{13}N , ^{15}O , ^{18}F , and for single photon emission computed tomography (SPECT) radiopharmaceuticals are $^{94\text{m}}\text{Tc}$, ^{201}Tl and ^{67}Ga . Other non-limiting examples of radionuclides include gamma emission isotopes. The person skilled in the art will understand that the radionuclides can also be used for therapeutic purposes.

Detectable reagents for CT imaging include, e.g., iodinated or brominated contrast media, e.g., iohalamate, iohexyl, diatrizoate, iopamidol, ethiodol and iopanoate. Gadolinium agents or gadopentate agents have also been reported to be of use as a CT contrast agent.

Detectable reagents for optical imaging include, e.g., fluorescein, a fluorescein derivative, indocyanine green, Oregon green, a derivative of Oregon green derivative, rhodamine green, a derivative of rhodamine green, an eosin, an erythrosin, Texas red, a derivative of Texas red, malachite green, nanogold sulfosuccinimidyl ester, cascade blue, a coumarin derivative, a naphthalene, a pyridyloxazole derivative, cascade yellow dye, dapoxyl dye, and various other fluorescent compounds, such as Cy3, Cy2, Cy5, the Alexa Fluor[®] fluorescent label family (Molecular Probes, Inc.), carboxyfluorescein (FAM) and fluorescein isothiocyanate (FITC).

In another embodiment, the detectable reagent is a protein. The term "protein", in the present context, refers to macromolecules consisting of one or more amino acid chains. Proteins can be bound to other proteins as well as to small substrate molecules. Non-limiting examples of proteins suitable for the purposes of the present invention include, without limitation, enzymes, fluorescent proteins, luminescent proteins and antigens.

Non-limiting examples of enzymes suitable for the invention include, without limitation, horseradish peroxidase (HRP) and alkaline phosphatase; and examples of suitable substrates include, without limitation, *p*-Nitrophenyl phosphate (PNPP), 2,2'-azinobis[3-ethylbenzothiazolin-6-sulfonic acid] (ABTS), *o*-phenylenediamine (OPD), and 3,3',5,5'-tetramethylbenzidine (TMB).

Non-limiting examples of antigens include, *inter alia*, tumor antigens, such as the carcinoembryonic antigen (CEA), HER2, prostate specific antigen (PSA) and tissue plasminogen activator and its recombinant variants, such as Activase®, as well as bacterial antigens, allergens, etc. It is understood that the antigens suitable for use in the present invention are indirectly detectable as a result of their capability of being specifically recognized by an antibody.

In another embodiment, the detectable reagent is a haptene. The term "haptene", in the present context, refers to a group of chemical compounds having a small molecular size (< 10,000 Da) which are antigenic but unable to induce by themselves an specific immune reaction. The chemical coupling of a haptene to a large immunogenic protein, called carrier, generates an haptene-immunogenic carrier conjugate which is able to induce a specific immune reaction. Non-limiting examples of haptenes include biotin (vitamin B7), digoxigenin, dinitrophenol (DNP) and nitro-iodophenol (NIP). It is understood that biotin is indirectly detectable as a result of its capability of being specifically recognized by avidin or variants thereof, such as streptavidin and neutravidin.

In another embodiment, the functional group is a drug. The term "drug", in the present context, refers to a chemical substance used in the treatment, cure or prevention of a disease or condition, such as a pathology characterized by an increase in expression of TLR-4 and/or an increase in activation of TLR-4. Particularly, the drug is a TLR-4 antagonist agent or an anti-inflammatory agent. Non-limiting examples of drugs are TLR-4 antagonists (e.g., naloxone, naltrexone, ibudilast, propentofylline, amitriptyline, ketotifen, cyclobenzaprine, mianserin and imipramine), anti-platelet drugs (e.g., aspirin and clopidogrel), anti-coagulants (e.g., heparin, acenocumarol, warfarin, dabigatran and rivaroxaban), and antioxidants (e.g., edaravone).

In an embodiment, the drug is a nucleic acid. Nucleic acids suitable as drugs in the context of the complex of the invention include antisense RNA, antisense DNA and small interfering RNA, which have the capability of silencing the expression of genes involved in a pathology characterized by an increase in expression of TLR-4 and/or an increase in activation of TLR-4, including, without

limitation, the *NFKB1*, *RIPK3*, *IFNB1*, *LY96 (MD-2)*, *IRF3*, *TLR3*, *TIRAP (Mal)*, *TICAM1 (TRIF)*, *RIPK1*, *TRAF6*, *CD14*, *TRAM*, *IKBKG (IKK-gamma)*, *IFNA1* and *TLR4* genes.

5 In an embodiment, the drug is a peptide, particularly, a peptide with the capability of binding to a target and of inducing or inhibiting cell signaling.

10 In another embodiment, the functional group is a nanoparticle. The term "nanoparticle", in the present context, refers to colloidal systems of the spherical type, rod type, polyhedron type, etc., or similar shapes, having a size less than 1 micrometer (μm), which are individually found or are found forming organized structures (dimers, trimers, tetrahedrons, etc.), dispersed in a fluid (aqueous solution). In a particular embodiment, the have a size less than 1 μm , generally comprised between 1 and 999 nanometers (nm), typically between 5 and 500 nm, particularly between about 10 and 150 nm. In a particular embodiment, the nanoparticles typically have a mean particle diameter ranging from 2 to 50 nm, particularly from 5 to 20 nm, more particularly of 13 nm.

15 Nanoparticles suitable for use in the present invention include polymeric nanoparticles, lipid nanoparticles and metal nanoparticles.

20 Polymeric nanoparticles are formed by a polymeric matrix which is attached to the aptamer. Non-limiting examples of biocompatible polymers that can be useful in the polymeric nanoparticles according to the present invention include polyethylenes, polycarbonates, polyanhydrides, polyhydroxyacids, polypropylfumerates, polycaprolactones, polyamides, polyacetals, polyethers, polyesters, poly(orthoesters), polycyanoacrylates, polyvinyl alcohols, polyurethanes, polyphosphazenes, polyacrylates, polymethacrylates, polycyanoacrylates, polyureas, polystyrenes, or polyamines, polyglutamate, dextran, polyanhydrides, polyurethanes, polymethacrylates, polyacrylates or polycyanoacrylates, polydioxanone (PDO), polyhydroxyalkanoate, polyhydroxybutyrate, poly(glycerol sebacate), polyglycolide, polylactide, PLGA, polycaprolactone or combinations thereof.

30 Alternatively, the nanoparticles can be lipid nanoparticles such as a liposome or a micelle. The vesicle-forming lipids are particularly lipids having two hydrocarbon chains, typically acyl chains, and a head group, either polar or nonpolar. There are a variety of synthetic vesicle-forming lipids and naturally-occurring vesicle-forming lipids, including the phospholipids, such as phosphatidylcholine, phosphatidylethanolamine, phosphatidic acid, phosphatidylinositol, and sphingomyelin, where the two-hydrocarbon chains are typically between about 14-22 carbon atoms in length, and either saturated or having varying degrees of unsaturation. Other suitable lipids include phospholipids, sphingolipids, glycolipids, and sterols, such as cholesterol.

Polymeric and lipidic nanoparticles can additionally include a coating of an amphiphilic compound that surrounds the polymeric material forming a shell for the particle or a stealth material that can allow

the particles to evade recognition by immune system components and increase particle circulation half-life.

Alternatively, the nanoparticles can be metal nanoparticles. The term "metal nanoparticle" refers to a nanoparticle comprising a metal and showing the optical property known as the surface plasmon phenomenon, i.e., a plasmonic metal. In an embodiment, said metal is selected from the group consisting of gold, silver, copper, aluminum, platinum, iron, cobalt, palladium and combinations thereof. In a particular embodiment, the nanoparticle is a metal nanoparticle. A particular embodiment of metal nanoparticles is a core-shell nanoparticle, which contains a metal core and a porous shell, e.g., magnetic mesoporous silica nanoparticles. Thus, in a particular embodiment, the nanoparticle is a magnetic mesoporous silica nanoparticle.

The nanoparticles can be functionalized by adding a coating on its surface. For biological applications, the surface coating should be polar to give high aqueous solubility and prevent nanoparticle aggregation. In serum or on the cell surface, highly charged coatings promote non-specific binding, whereas polyethylene glycol linked to terminal hydroxyl or methoxy groups repel non-specific interactions.

Aptamers of the present invention can be linked to nanoparticles ideally by a covalent link, particularly on the nanoparticle surface. Particularly, aptamers are present in a controlled number per nanoparticle.

Uses of the aptamers and complexes

Medical uses of the aptamers and complexes

As described previously, the examples of the present invention provide evidence of the anti-tumor activity of the aptamers described herein (EXAMPLE 7). Further, the invention also demonstrates the effect of the aptamers on the experimental autoimmune neuritis (EAN) model, a representative animal model of an inflammatory autoimmune neuropathy disorder and Guillain-Barré syndrome (GBS) (EXAMPLE 8), and also on the experimental autoimmune encephalomyelitis (EAE) model, a preclinical model of multiple sclerosis (EXAMPLE 12). Thus, the present invention describes for the first time the positive effect of the aptamers of the invention (e.g., SEQ ID NO: 1-6) in the treatment, prevention and/or amelioration of a disease.

Accordingly, another aspect of the present invention relates to an aptamer or a complex of the invention for use as a medicament.

The invention also encompasses the aptamers and complexes described herein (e.g., SEQ ID NO: 1-6) for use in the treatment of a pathological condition or disease susceptible to amelioration by

inhibition of TLR-4 receptor, comprising administering a therapeutically effective amount of at least one aptamer or complex described herein.

Use in the treatment of cancer

5

An aspect of the present invention relates to an aptamer or a complex described herein (e.g., SEQ ID NO: 1-6) for use in the treatment of cancer.

10

In some embodiments, the cancer can be, but it is not limited to breast cancer, bladder cancer, colorectal cancer, kidney cancer, lung cancer, non-small cell lung cancer (NSCLC), squamous cell carcinoma, lymphoma, melanoma, oral or oropharyngeal cancer, nasopharyngeal carcinoma, pancreatic cancer, prostate cancer, thyroid cancer, uterine cancer, adenocarcinoma, endometrial cancer, ovarian cancer, cervical cancer, renal cancer, glioblastoma, skin cancer, thymic carcinoma, leukemia, gastrointestinal cancer, liver cancer, esophageal cancer, gastric cancer, head and neck cancer, multiple myeloma cancer, neuroblastoma, or cholangiocarcinoma.

15

In an embodiment, the cancer is selected from the group consisting of lung cancer, non-small cell lung cancer, squamous cell carcinoma, prostate cancer, breast cancer, melanoma, colorectal cancer, head and neck cancer, nasopharyngeal cancer, uterine cancer, renal cancer, thymic carcinoma and leukemia. In a particular embodiment, the cancer is breast cancer.

20

In some embodiments, the breast cancer is selected from the group consisting of ductal carcinoma in situ (DCIS), invasive ductal carcinoma (IDC), invasive lobular carcinoma (ILC), inflammatory breast cancer (IBC), hormone receptor-positive breast cancer (HR+) (e.g., estrogen receptor (ER)+/progesterone receptor (PR)+, ER+/PR-, ER-/PR+), HER2-positive breast cancer (HER2+ or ERBB2+) (e.g., ER+/PR+/HER2+, ER-/PR-/HER2+), triple-negative breast cancer (TNBC) (i.e., ER-/PR-/HER2-), Paget's disease of the breast, angiosarcoma, phyllodes tumor, and metastatic breast cancer.

25

Alternatively, the invention also encompasses a method of treatment of cancer (e.g., breast cancer), comprising administering to a subject in need thereof a therapeutically effective amount of an aptamer or a complex of the invention.

30

TLR-4 overexpression can contribute to resistance to chemotherapy, e.g., resistance to paclitaxel in ovary cancer and resistance to siRNA therapy in prostate cancer. TLR-4 signaling has also been linked to resistance to chemotherapy in liver cancer. Accordingly, the methods and compositions described herein can be used to reduce, prevent, or reverse resistance to chemotherapy in cancer patients.

35

TLR-4 signaling in immune and inflammatory cells in a tumor microenvironment lead to the

40

production of inflammatory cytokines, which can result in further polarization of tumor associated macrophages, conversion of fibroblasts into tumor-promoter cancer associated fibroblasts, conversion of dendritic cells into tumor-associated DCs, and activation of pro-tumorigenic function of immature myeloid cells. In some embodiments, the uses/methods and aptamers/complexes of the present invention can be used to (i) inhibit or reduce the production of inflammatory cytokines, (ii) reduce or inhibit polarization of tumor associated macrophages, (iii) reduce or inhibit conversion of fibroblasts into tumor-promoter cancer associated fibroblasts, (iv) reduce or inhibit conversion of dendritic cells into tumor-associated DCs, (v) reduce or inhibit activation of pro-tumorigenic function of immature myeloid cells, or (vi) any combination thereof.

10

In some embodiments, the administration of the aptamer or complex of the invention results in at least one outcome (i.e., effect) selected, but not limited to, from the group consisting of:

- reduction in tumor progression;
- delayed disease progression (or slow progression);
- 15 - tumor shrinkage;
- tumor size reduction (e.g., diameter, perimeter);
- reduction or prevention of tumor growth;
- inhibition or reduction of angiogenesis;
- inhibition or reduction of tumor invasion;
- 20 - inhibition or reduction of metastasis;
- improved survival rate (e.g., increase in survival);
- reduction in side effects;
- increase in quality of life;
- improved prognosis;
- 25 - enhanced response to therapy;
- improved disease-free survival;
- decrease in TLR-4 protein levels; and
- decrease in mRNA TLR-4 levels.

30

In a particular embodiment, the aptamer for use in the treatment of cancer (e.g., breast cancer) is selected from the group consisting of SEQ ID NO: 1-6. More particularly, the aptamer is SEQ ID NO: 2 (ApSION 2) or SEQ ID NO: 4 (ApSION 4).

35

Another aspect relates to SEQ ID NO: 7 (ApTOLL) for the treatment of cancer, particularly breast cancer.

Use in the treatment of a neuromuscular or neurodegenerative disease or condition

40

Further, an aspect of the present invention relates to an aptamer or a complex described herein (e.g., SEQ ID NO: 1-6) for use in the treatment of a neuromuscular or neurodegenerative disease or

condition. In a particular embodiment, the aptamers of the present invention are for use in ameliorating or improving at least a symptom or sequelae of a neuromuscular or neurodegenerative disease or condition, wherein the aptamer is administered during, prior, or after the onset of the neuromuscular or neurodegenerative disease or condition.

5

In a particular embodiment, the disease is a neuromuscular disease. Non-limiting examples of neuromuscular diseases are amyotrophic lateral sclerosis (ALS), muscular dystrophy (e.g., Duchenne muscular dystrophy, Becker muscular dystrophy), spinal muscular atrophy (SMA), myasthenia gravis, an inflammatory autoimmune neuropathy (e.g., Guillain-Barré syndrome), Charcot-Marie-Tooth disease, peripheral neuropathy, polymyositis, inclusion body myositis, or Lambert-Eaton myasthenic syndrome. In a particular embodiment, the neuromuscular disease is an inflammatory autoimmune neuropathy.

10

In another embodiment, the disease is a neurodegenerative disease. Non-limiting examples of neurodegenerative diseases are Parkinson's disease, Huntington's disease, Alzheimer's disease, multiple sclerosis, amyloid lateral sclerosis (ALS), vascular dementia disease, frontotemporal dementia, spinocerebellar ataxia, progressive supranuclear palsy, corticobasal degeneration, or Creutzfeldt-Jakob disease. In a particular embodiment, the neurodegenerative disease is multiple sclerosis.

15

In some embodiments, the neuromuscular or neurodegenerative disease or condition is selected from the group consisting of an inflammatory autoimmune neuropathy, amyotrophic lateral sclerosis (ALS), Parkinson's disease, Huntington's disease, Alzheimer's disease, and vascular dementia disease.

20

In some embodiments, the neuromuscular or neurodegenerative disease or condition is an immune-mediated neurological disorder or an autoimmune neurological condition. In a particular embodiment, the immune-mediated neurological disorder or the autoimmune neurological condition is an inflammatory autoimmune neuropathy or multiple sclerosis.

25

Further, an aspect of the present invention relates to an aptamer or a complex described herein (e.g., SEQ ID NO: 1-6) for use in the treatment of an inflammatory autoimmune neuropathy disorder. Inflammatory autoimmune neuropathy refers to a group of disorders in which the body's immune system mistakenly attacks the peripheral nerves, resulting in inflammation and damage.

30

In some embodiments, the inflammatory autoimmune neuropathy can be, but it is not limited to Guillain-Barré Syndrome (GBS), Chronic Inflammatory Demyelinating Polyneuropathy (CIDP), Multifocal Motor Neuropathy (MMN), Vasculitic Neuropathy, Sensory Ganglionopathy, or Paraneoplastic Neuropathy. Particularly, the inflammatory autoimmune neuropathy is Guillain-Barré syndrome (GBS). GBS is an acute inflammatory autoimmune neuropathy that affects the peripheral nervous system.

35

It is often triggered by an infection or vaccination, and usually develops rapidly causing muscle

40

weakness, paralysis, and respiratory failure.

Alternatively, the invention also encompasses a method of treatment of inflammatory autoimmune neuropathy disorder as described herein (particularly Guillain-Barré syndrome), comprising administering to a subject in need thereof a therapeutically effective amount of an aptamer or a complex of the invention.

In some embodiments, the administration of the aptamer or complex of the invention results in at least one outcome (i.e., effect) selected, but not limited to, from the group consisting of:

- improvement in neurological symptoms (e.g., reduction in symptoms such as muscle weakness, sensory loss, and pain);
- stabilization or improvement in nerve conduction studies (NCS) (NCS parameters can be nerve conduction velocity, amplitude, and latency);
- reduction in disease activity (e.g., decrease in inflammatory markers such as C-reactive protein (CRP) or erythrocyte sedimentation rate (ESR)); and
- improvement in quality of life (e.g., pain, disability, and overall function).

Particularly, the administration of the aptamer or complex of the invention results in at least one outcome (i.e., effect) selected, but not limited to, from the group consisting of:

- increase of neuromotor performance;
- increase of neuromuscular performance;
- increase of electrophysiological performance;
- increase of nerve conduction amplitude and velocity;
- decrease of plasma inflammatory cytokines (e.g., IL-6 or TNF- α);
- decrease of TLR-4 protein levels; and
- decrease of mRNA TLR-4 levels.

In a particular embodiment, the aptamer for use in the treatment of an inflammatory autoimmune neuropathy (e.g., Guillain-Barré syndrome) is selected from the group consisting of SEQ ID NO: 1-6. More particularly, the aptamer is SEQ ID NO: 2 (ApSION 2) or SEQ ID NO: 4 (ApSION 4), and even more particularly SEQ ID NO: 4.

In a particular embodiment, the aptamer for use in the treatment of multiple sclerosis is selected from the group consisting of SEQ ID NO: 1-6. More particularly, the aptamer is SEQ ID NO: 2 or SEQ ID NO: 4, and even more particularly SEQ ID NO: 2.

Another aspects relates to SEQ ID NO: 7 (ApTOLL) for the treatment of an inflammatory autoimmune neuropathy disorder, particularly Guillain-Barré syndrome.

Use in the treatment of other diseases

As described above, the aptamers of the present inventions are capable of specifically binding to and inhibiting TLR-4, thus, it is plausible that the aptamers can be used in the treatment of a pathology characterized by an increase in expression of TLR-4 and/or an increase in activation of TLR-4, i.e. a TLR-4 mediated disease.

Thus, an aspect of the invention relates to the aptamers/complexes of the invention (e.g., SEQ ID NO: 1-6) for use in the treatment of a TLR-4 mediated disease. In some embodiments, TLR-4 mediated diseases and conditions comprise, e.g., acute diseases and conditions such as enterocolitis, influenza, ischemic stroke, sepsis, renal ischemia-reperfusion, liver ischemia-reperfusion, intracerebral hemorrhage, or myocardial ischemia; sub-acute diseases and conditions such as multiple sclerosis, addiction withdrawal, adenomyosis, keratitis, or pulmonary inflammation; and chronic diseases and conditions such as rheumatoid arthritis, atherosclerosis, asthma, lupus, osteoporosis, transplant rejection, dermatitis, psoriasis, obesity, type II diabetes, neuropathic pain, hypertension, RLA, aortic aneurysm, diffuse axonal injury, or chronic pain. Other TLR-4 mediated diseases can be hepatic steatosis, insulin resistance, or intrauterine infection leading to uterine smooth muscle contraction, among others.

In some embodiments, the disease is an autoimmune inflammatory disease, such as human systemic sclerosis (SSc), rheumatoid arthritis, systemic lupus erythematosus, Sjogren's syndrome, psoriasis, multiple sclerosis, or autoimmune diabetes, or fibrosis, e.g., dermal or lung fibrosis.

In a particular embodiment, the pathology characterized by an increase in expression of TLR-4 and/or an increase in activation of TLR-4 is selected from the group consisting of, *inter alia*, stroke (e.g., ischemic stroke, hemorrhagic stroke, hemorrhagic transformation, transient ischemic attack (TIA)), acute cardiac infarction (e.g., acute myocardial infarction), sepsis, atherosclerosis, multiple sclerosis, rheumatoid arthritis, a retinal degenerative disease (e.g., age-related macular degeneration, Stargardt disease, retinitis pigmentosa, choroideremia, Leber congenital amaurosis, retinosis juvenile, Usher disease, Bardet Biedl), and drug addiction.

In a particular embodiment, the aptamers/complexes of the present invention are for use in the treatment of acute cardiac infarction, wherein the treatment comprises improving cardiac function after acute cardiac infarction, wherein the aptamer is administered after the acute cardiac infarction, and wherein the administration of the aptamer results in an improvement of cardiac function. Alternatively, the aptamers of the present invention are for use in recovery of cardiac function in a subject having suffered acute myocardial infarction, and wherein the administration of the aptamer results in a recovery of cardiac function. Alternatively, the invention relates to a method of improving cardiac function after cardiac infarction in a subject in need thereof, the method comprising administering an aptamer of the present invention to the subject after the cardiac infarction.

Examples of TLR-4 mediated diseases and their outcomes are included e.g., in WO2015/197706A1, WO2020/230108A1 and WO2020/230109A1, which are herein incorporated by reference in their entirety.

5 *In vitro* uses and methods of the aptamers and complexes

The present invention also contemplates *in vitro* uses of the aptamers and of the complexes described herein, for detecting TLR-4. Therefore, another aspect of the invention refers to an *in vitro* use of an aptamer or a complex as described herein for detecting TLR-4.

10

Further, the present invention is also directed to *in vitro* uses of the aptamers and of the complexes described herein, for inhibiting TLR-4. Therefore, another aspect of the invention relates to an *in vitro* use of an aptamer or a complex as described herein for inhibiting TLR-4.

- 15 The capability of an aptamer according to the invention of binding specifically to TLR-4 can be exploited for the indirect detection of TLR-4 through the aptamer. Further, the complex described herein is particularly advantageous for detecting TLR-4, since the detectable reagent attached to the aptamer enables the detection of the aptamer when it is bound to TLR-4. The technique used for detecting TLR-4 will depend on the type of detectable reagent, being techniques based, e.g., on fluo-
- 20 rimetry, colorimetry or radioactivity.

For this purpose, the person skilled in the art will recognize that subsequent detection of the aptamer/complex is required. Aptamer detection techniques are well-known in the art and include, e.g., the use of antibodies or probes specific for the aptamer. Therefore, once the aptamer according to

25 the invention is bound to TLR-4, an antibody or probe specific for the aptamer, which in turn can be labeled with a detectable reagent, or which can be detected indirectly by means of a secondary antibody or probe, would be applied. The technique used for detecting TLR-4 will then depend on the type of detectable reagent, being techniques based, e.g., on fluorimetry, colorimetry or radioactivity.

30

The detection of TLR-4 with the aptamer/complex of the invention can be carried out by means of *in vitro* binding assays, such as the enzyme-linked oligonucleotide assay (ELONA), the enzyme-linked aptamer sorbent assay (ELASA), precipitation and quantitative PCR (qPCR), gel mobility shift assay, Western Blotting, surface plasmon resonance (SPR), kinetic capillary electrophoresis, the fluores-

35 cence binding assay, aptahistochemistry, aptacytochemistry, fluorescence microscopy or flow cytometry.

In an embodiment, the detection of TLR-4 is performed by means of a method selected from the group consisting of ELONA, aptacytochemistry, aptahistochemistry and flow cytometry. The person

skilled in the art will recognize that these techniques can be adapted for exchanging the detection antibody for a probe specific for the aptamer.

Alternatively, another aspect of the present invention relates to an *in vitro* method for the detection of TLR-4 in a sample comprising:

- 5 (i) contacting the sample with an aptamer or a complex as described herein;
 - (ii) separating the aptamer or complex not bound to TLR-4; and
 - (iii) detecting the presence of the aptamer or complex bound to the TLR-4 present in the sample.
- 10 In another aspect, the present invention relates to an *in vitro* method for inhibiting TLR-4 in a sample, which comprises contacting a sample comprising TLR-4 with an aptamer or a complex described herein in conditions suitable for inhibiting TLR-4.

The term "sample" or "biological sample", in the context of the present invention, refers to a cell
15 culture or to isolated biological material from a subject. The biological sample can contain any biological material suitable for detecting the desired biomarker and can comprise cells and/or non-cellular material from the subject. The sample can be isolated from any suitable tissue or biological fluid such as, e.g., blood, plasma, serum, urine, cerebrospinal fluid, heart, brain. The samples used for the detection of TLR-4 are particularly biological fluids. In a particular embodiment, the biological
20 sample is from a human subject.

The aptamer or complex according to the invention is applied on the sample in a buffer suitable for allowing the binding of the aptamer/complex to the TLR-4 molecules that can be present in the sample. The aptamer/complex is incubated with the sample at a suitable temperature and for a time
25 sufficient for allowing the binding of the aptamer/complex to the TLR-4 molecules, and then the sample is washed to remove the aptamer/complex molecules that have not bound to TLR-4. Then, the presence of the aptamer/complex bound to the TLR-4 present in the sample is detected.

The *in vitro* method of the invention can be carried out as part of detection techniques mentioned
30 above, such as ELONA, ELASA, precipitation and qPCR, gel mobility shift assay, Western Blotting, surface plasmon resonance, kinetic capillary electrophoresis, fluorescence binding assay, aptahistochemistry, aptacytochemistry, fluorescence microscopy or flow cytometry.

In another aspect, the invention relates to the use of a complex according to the invention for *in vivo*
35 imaging of a cell, tissue or organ which express TLR-4, wherein the complex comprises one or more aptamers according to the invention and a functional group, said functional group being a detectable moiety. Suitable detectable moieties for use in the *in vivo* imaging methods according to the invention have been described above in the context of the complex of the invention and include, without limitation, a radionuclide, a fluorophore, a contrast media, a protein and an haptene.

Pharmaceutical compositions

For the administration to a subject in need of an aptamer or complex of the invention, the aptamers or complexes can be formulated in suitable pharmaceutical compositions.

5

In another aspect, the present invention relates to a pharmaceutical composition comprising an aptamer or a complex of the invention described herein (e.g., SEQ ID NO: 1-6) together with at least a pharmaceutically acceptable excipient, carrier, or solvent. Excipients are selected, without limitation, from the group comprising: fillers/diluents/bulking agents, binders, antiadherents, disintegrants, coatings, anti-caking agents, antioxidants, lubricants, sweeteners, flavors, colors, or tensides.

10

The term "pharmaceutically acceptable" is art-recognized, and includes excipients, compounds, materials, compositions, carriers, vehicles and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of a subject (e.g., human or animal) without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio. Each carrier, excipient, etc. must also be "acceptable" in the sense of being compatible with the other ingredients of the formulation. Suitable carriers, excipients, etc. can be found in standard pharmaceutical texts.

15

In an embodiment, the aptamer is formulated in PBS-MgCl₂. In an embodiment, the formulation comprises sodium chloride, potassium chloride, disodium hydrogen phosphate dehydrate, and potassium dihydrogen phosphate to generate a phosphate-buffered solution at pH 7.4, comprising magnesium chloride hexahydrate. This buffer solution and conditions support the aptamer structure and its biological activity.

20

The pharmaceutical compositions provided by the present invention can be administered to a subject by means of any suitable administration route, such as, e.g., by parenteral route. The term "parenteral", in the context of the present invention, includes the intravenous, intraperitoneal, intramuscular, intraarterial, or subcutaneous administration. Particularly, the composition is in intravenous form.

25

In some embodiments, the pharmaceutical composition can be administered via intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal and intrasternal injection and infusion. In specific embodiments, the pharmaceutical composition is administered intravenously or intraarterially, e.g., via infusion or via bolus. In some embodiments, the administration is via a slow bolus, i.e., the dose is administered via injection lasting about: 1 minute, 2 min, 3 min, 4 min, 5 min, 6 min, 7 min, 8 min, 9 min, 10 min, 11 min, 12 min, 13 min, 14 min, or 15 min.

30

35

Particularly, the dosage is provided by means of injections, more particularly intravenous or subcutaneous injections, depending in part on whether the administration is acute or chronic.

Combinations with other therapeutic agents

5

The invention is also directed to combinations comprising at least one aptamer or complex described herein (e.g., SEQ ID NO: 1-6) and one or more other therapeutic or biologically active agents or treatments. The aptamer or complex and the other therapeutic agent can be formulated for a separate, sequential, concomitant administration or in a mixture in a single pharmaceutical composition.

10

Accordingly, in some embodiments, the aptamer or complex of the present invention is combined with one or more therapeutic agent or treatment. Particular embodiments of therapeutic agents are described hereinafter.

15 In particular embodiments, the other therapeutic agent or treatment is an anti-tumor agent, e.g., a chemotherapeutic agent, a targeted therapy agent (e.g., monoclonal antibody such as trastuzumab or bevacizumab), an immunotherapy agent (e.g., anti-PD1, anti-PDL1, anti-CTLA4 checkpoint inhibitors) or radiotherapy.

20 Non-limiting examples of chemotherapeutic agents are: bifunctional alkylator (e.g., Cyclophosphamide, Mechlorethamine, Chlorambucil or Melphalan); monofunctional alkylator (e.g., Dacarbazine(DTIC), Nitrosoureas or Temozolomide); anthracycline (e.g., Daunorubicin, Doxorubicin, Epirubicin, Idarubicin, Mitoxantrone or Valrubicin); taxane (e.g., Paclitaxel, Docetaxel, Nab-Paclitaxel or Taxotere); epothilone (e.g., patupilone, sagopilone or ixabepilone); deacetylase inhibitor (e.g., Vorinostat or Romidepsin); inhibitor of topoisomerase I (e.g., Irinotecan or Topotecan); inhibitor of topoisomerase II (e.g., Etoposide, Teniposide or Tafluposide); kinase inhibitor (e.g., Bortezomib, Erlotinib, Gefitinib, Imatinib, Vemurafenib or Vismodegib); nucleotide analog and/or precursor analog (e.g., Azacitidine, Azathioprine, Capecitabine, Cytarabine, Doxifluridine, Fluorouracil, Gemcitabine, Hydroxyurea, Mercaptopurine, Methotrexate or Tioguanine); peptide antibiotic (e.g., Bleomycin or Actinomycin); platinum-based agent (e.g., Carboplatin, Cisplatin or Oxaliplatin); retinoid (e.g., Tretinoin, Alitretinoin or Bexarotene); and vinca alkaloid and derivative (e.g., Vinblastine, Vincristine, Vindesine or Vinorelbine).

35 The most commonly used therapeutic agents for hormone receptor-positive breast cancer (HR+) are endocrine therapy such as selective estrogen receptor modulators (SERMs) (e.g., tamoxifen and raloxifene), aromatase inhibitors (AIs) (e.g., letrozole, anastrozole, exemestane), or luteinizing hormone-releasing hormone (LHRH) agonists (e.g., goserelin, leuprolide); chemotherapy (e.g., adriamycin / cyclophosphamide (AC), adriamycin / cyclophosphamide / paclitaxel (AC-T), or docetaxel / cyclophosphamide (TC)); and targeted therapies (e.g., CDK4/6 inhibitors such as palbociclib, ribociclib, abemaciclib).

40

Therapeutic agents for HER2-positive breast cancer (HER2+ or ERBB2+) can be targeted therapies directed to HER2 protein such as trastuzumab, pertuzumab, T-DM1 or neratinib; in combination with chemotherapy (e.g., paclitaxel / trastuzumab (TH), adriamycin / cyclophosphamide / paclitaxel, 5 trastuzumab ± pertuzumab (AC-TH±P), or docetaxel / carboplatin / trastuzumab ± pertuzumab (THC±P)); and endocrine therapy if the hormonal receptor is positive (e.g., tamoxifen, letrozole, anastrozole, or exemestane).

Non-limiting therapeutic agents for triple-negative breast cancer (TNBC) are chemotherapy (e.g., 10 anthracyclines, taxanes, platinum agents, or others), or immunotherapy such as checkpoint inhibitors (e.g., pembrolizumab or atezolizumab) which e.g., can be used in combination with chemotherapy for patients with advanced or metastatic TNBC that expresses specific biomarkers like PD-L1.

Regarding the treatment of inflammatory autoimmune neuropathies, it typically involves immunosuppressive therapy to reduce inflammation and modulate the immune response. The choice of treatment depends on the specific type of neuropathy and the severity of symptoms. 15

Accordingly, in some embodiments, the other therapeutic agent or treatment is an immunosuppressive therapy, such as a corticosteroid (e.g., prednisone or methylprednisolone), an intravenous immunoglobulin, plasmapheresis, an immunosuppressive drug (e.g., azathioprine, cyclophosphamide, 20 mycophenolate mofetil or methotrexate), or a biological agent (e.g., rituximab, tocilizumab, or eculizumab).

Methods of manufacture and formulation

25 Another aspect of the present invention provides methods of making the aptamers or complexes of the present invention (e.g., SEQ ID NO: 1-6). The aptamers can be chemically synthesized as disclosed herein (EXAMPLE 1) and by a method known *per se* in the art. Chemical modifications of nucleotides can be introduced during nucleotide synthesis or added post-synthesis, either enzymatically or chemically. 30

Non-limiting examples of techniques for the production of aptamers include enzymatic techniques, such as transcription, recombinant expression systems and standard solid phase (or solution phase) chemical synthesis, all commercially available. 35

In a particular embodiment, the method of synthesis of the aptamers or complexes of the present invention is the chemical synthesis on solid-phase supports using phosphoramidite, H-phosphonate or phosphodiester derivatives. In these methods, the nucleotide at the 3'-end is attached to a solid support and the support is subject to a cycle of reactions including detritylation, coupling, capping 40 and oxidation that introduce one by one the nucleotides of the aptamer until reaching the desired

sequence. At the end of the assembling process, the support is treated with ammonia or similar to release the desired aptamer that is purified from truncated sequences generated in the synthetic process. Each nucleotide monomer unit is functionalized with the following functions: the dimethoxytrityl (DMT) group is used for the protection of the hydroxyl at the 5'-position (except for the inverted nucleotides that have the DMT group at the 3'-end) and the 2-cyanoethyl-*N,N*-diisopropylamino phosphoramidite at the 3'-end. The amino groups of the nucleobases are protected with the benzoyl or isobutyryl groups or other. Modified nucleotide units carry similar protecting groups and they are added to the sequence using similar protocols.

As used herein, the term "synthesizing" refers to the assembling the aptamer using polynucleotide synthesis methods known in the art. The term synthesizing also encompasses the assembly of conjugates or complexes that comprise an aptamer of the present invention and at least one biological active molecule (e.g., a small molecule drug covalently or non-covalently attached to the aptamer). For example, peptide or small molecule components can be prepared recombinantly, chemically, or enzymatically and subsequently conjugated to the aptamer in one or more synthesis steps (e.g., conjugation of a linker to an aptamer of the present invention followed by conjugation of a small molecule to the linker).

The aptamers of the present invention can be purified, e.g., via filtration, to remove contaminants and/or to generate an uniform population of aptamers. In some embodiments, the manufacture of the aptamers of the present invention comprise lyophilization or any other form of dry storage suitable for reconstitution. In some embodiments, the preparation of the aptamer in a dry form takes place after combination of the aptamer with a biologically active molecule (e.g., a small molecule drug), i.e., both therapeutic agents can be co-lyophilized.

In some embodiments, the method of preparing a composition comprising an aptamer of the present invention with a biologically active molecule (e.g., a small molecule drug) comprises mixing the aptamer with the biologically active molecule in solution. In some embodiments, after combination of the aptamer and the biologically active molecule in solution, the resulting solution is lyophilized or dried. In some embodiments, the combination of the aptamer and the biologically active molecule is conducted in dry form.

The present invention also provides formulations comprising aptamers of the present invention (e.g., SEQ ID NO: 1-6). The aptamer is combined with a solution comprising previously filtered excipients. After a structuration stage, the solution comprising the aptamer and excipients is subject to two filtration steps, transferred to vials, and lyophilized.

The structuration step is a critical step in the preparation of the aptamer. The structuration process comprises dissolving the aptamer in an appropriate solvent free of nucleases. In some embodiments, the solvent comprises a divalent ion. In some embodiments, the divalent ion is Mg^{2+} . In some

embodiments, the solvent is phosphate buffered saline (PBS) comprising MgCl_2 . In some embodiments, the solvent is PBS comprising 1 mM MgCl_2 . After the aptamer has been dissolved, it is heated up to a denaturing temperature (e.g., 95 °C) for a short period of time (e.g., approximately 10 minutes) followed by rapid cooling (e.g., at 0 °C in ice, e.g., during approximately 10 minutes). After synthesis, aptamers of the present invention are linear (e.g., SEQ ID NO: 1-6). Increasing the temperature fully linearizes the aptamer, whereas the subsequent cooling down correctly folds the aptamer, resulting in a functional aptamer.

The process of manufacture of the aptamers of the present invention comprises two lyophilization steps. In a first step, the synthesized aptamer is lyophilized rendering the API. Then, the API is reconstituted in a properly solution (e.g., an aptamer of the present invention in PBS-1mM MgCl_2) to conduct the structuration following by a second lyophilization. This second lyophilization keeps the proper structure of the aptamer over the time.

In some embodiments, the aptamers of the present invention are formulated in doses comprising between 0.5 mg and 10 mg of aptamer, e.g., structured and lyophilized aptamer. In other embodiments, the aptamers are formulated in doses comprising at least about: 0.5 mg, 1 mg, 2 mg, 3 mg, 4 mg, 5 mg, 6 mg, 7 mg, 8 mg, 9 mg, or 10 mg of aptamer.

In some embodiments, the aptamer of the present invention can be formulated, e.g., in nanoparticles such as polymeric nanoparticles, lipid nanoparticles (e.g., liposomes or micelles), or metal nanoparticles, comprising the aptamers of the present invention covalently or non-covalently attached to the nanoparticle (e.g., encapsulated in the nanoparticle). See, e.g., U.S. Patent No. 10,196,642, which is herein incorporated by reference in its entirety.

As described above, the aptamers of the present invention can be covalently or non-covalently attached to another molecule forming a complex, e.g., to a biologically active molecule and/or to a nanoparticle. Covalent attachment between an aptamer and another molecule can be carried out by means of conjugation techniques that are well-known by the person skilled in the art. The result is a covalent bond between the aptamer and a biologically active molecule and/or to a nanoparticle or its components. The conjugation can involve binding of primary amines of the 3' or 5' ends of the aptamer to the functional group during chemical synthesis of the aptamer.

Conjugation can also be done by means of conventional cross-linking reactions, having the advantage of the much greater chemical reactivity of primary alkyl-amine labels with respect to the aryl amines of the nucleotides themselves. Methods of conjugation are well-known in the art and are based on the use of cross-linking reagents. The cross-linking reagents contain at least two reactive groups which target groups such as primary amines, sulfhydryls, aldehydes, carboxyls, hydroxyls, azides, and so on and so forth, in the biologically active molecule and/or nanoparticle to be conjugated to an aptamer of the present invention.

The cross-linking agents differ in their chemical specificity, spacer arm length, spacer arm composition, cleavage spacer arm, and structure. For example, conjugation of biologically active molecules and/or nanoparticles or their components to aptamer of the present invention can be carried out directly or through a linking moiety, through one or more non-functional groups in the aptamer and/or the functional group, such as amine, carboxyl, phenyl, thiol or hydroxyl groups. More selective bonds can be achieved by means of the use of a heterobifunctional linker. It is possible to use conventional linkers, such as diisocyanates, diisothiocyanates, bis (hydroxysuccinimide) esters, carbodiimides, maleimide-hydroxysuccinimide esters, glutaraldehyde and the like, or hydrazines and hydrazides, such as 4-(4-N-maleimidophenyl) butyric acid hydrazide (MPBH).

In some embodiments, conjugation can take place subsequently to the generation of the aptamer of the present invention by recombinant or enzymatic methods.

In some embodiments, the aptamers of the present invention are formulated in vials, wherein each dose vial comprises about: 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, or 10 mg of aptamer of the present invention per vial. In an embodiment, each dose vial comprises 7 mg of aptamer per vial. In some embodiments, the content of the vials is lyophilized aptamer.

EXAMPLES

CHARACTERIZATION OF APTOLL AND APSION 1-6

EXAMPLE 1: Aptamer synthesis and purification

1.1 Aptamer synthesis

Oligonucleotides (see Table 1) were synthesized on a H-8 DNA/RNA synthesizer (K&A Laboratories, Germany) in a 1- μ mol scale using the appropriate controlled pore glass (CPG) supports and the 5'-O-dimethoxytrityl(DMT)-protected 2-cyanoethyl phosphoramidites at 0.1 M in acetonitrile. For the detritylation step 3 % trichloroacetic acid in dichloromethane (Applied Biosystems 401272) was used. The coupling agent was 5-benzylthio-1H-tetrazole (BTT) at 0.3 M in acetonitrile (Link Technologies LK3162-D200) with a coupling time of 20 seconds. The capping step was done using two different capping solutions, the capping A with pyridine/Acetic anhydride 8:1 (Link Technologies LK4110-D200) and the capping B with 10 % methylimidazole in tetrahydrofuran (Link Technologies LK4120-D200). Finally, for the oxidation step an iodine solution of 0.02 M with 0.4 % pyridine (Link Technologies LK4132-D200) was used. The last DMT group was left on the 5'-end (DMT on mode). The phosphate and base-protecting groups and the oligonucleotide-solid support link were removed by treatment of the CPG support with concentrated ammonia at 55 °C for more than 6 hours.

Table 1. Oligonucleotide sequences.

Reference	Sequence (5'-3')
ApSION 1 (SEQ ID NO: 1)	GGfU GfUG fCfCA AfUA AAfC fCAfU AfUfC GfCfC GfCG fUfUA GfCA fUGfU AfCfU fCGG fUfUG GfCfC fCfUA AAfU AfCG AGiT
ApSION 2 (SEQ ID NO: 2)	GGeT GeTG eCeCA AeTA AAeC eCAeT AeTeC GeCeC GeCG eTeTA GeCA eTGeT AeCeT eCGG eTeTG GeCeC eCeTA AAeT AeCG AGiT
ApSION 3 (SEQ ID NO: 3)	GGIT GiTG ICICA AITA AAIC ICAIT AiTiC GiCiC GiCG ITITA GICA iTGiT AiCiT ICGG iTiTG GiCiC ICITA AAiT AICG AGiT
ApSION 4 (SEQ ID NO: 4)	GGmU GmUG mCmCA AmUA AAmC mCAmU AmUmC GmCmC GmCG mUmUA GmCA mUGmU AmCmU mCGG mUmUG GmCmC mCmUA AAmU AmCG AGiT
ApSION 5 (SEQ ID NO: 5)	GGT GTG mCmCA AmUA AAC CAmU AmUC GmCmC GCG mUmUA GCA mUGT AmCmU mCGG mUmUG GmCC CTA AAT ACG AGiT
ApSION 6 (SEQ ID NO: 6)	mCmCA AmUA AAmC mCAmU AmUmC GmCmC GmCG mUmUA GmCA mUGmU AmCmU mCGG mUmUG GmCiC
ApTOLL (SEQ ID NO: 7)	GGT GTG CCA ATA AAC CAT ATC GCC GCG TTA GCA TGT ACT CGG TTG GCC CTA AAT ACG AG

fU: 2'-fluoro-2'-deoxyuridine (2'-F-2'-dU); fC: 2'-fluoro-2'-deoxycytidine (2'-F-2'-dC); eT: 2'-O-methoxyethyl-5-methyluridine (2'-O-MOE-T); eC: 2'-O-methoxyethyl-5-methylcytosine (2'-O-MOE-meC); iT: LNA-T; IC: LNA-C; mU: 2'-O-methyluridine (2'-OMe-U); mC: 2'-O-methylcytidine (2'-OMe-C); iT: inverted-T; iC: inverted-C.

5

See also FIG. 32 that shows the pattern of chemical modifications along the sequence of the new aptamers ApSION 1-6, compared to ApTOLL sequence.

1.2 Cartridge purification

- 10 The ammonia solutions (1 mL) obtained after deprotection containing DMT-oligonucleotide were purified directly without evaporation of the ammonia. 1 mL of a 100 mg/mL NaCl solution was added to the ammonia solution to a final volume of 2 mL. Previously, the Glen-Pack DNA cartridge was washed with 0.5 mL of acetonitrile followed by 1 mL of 2M triethylammonium acetate (TEAA). The salty ammonia solution containing the DMT-oligonucleotide was loaded to the purification cartridge recovering the washes. Then the cartridge was washed twice with 1 mL a 5% acetonitrile in
- 15 100mg/mL sodium chloride (salt wash solution) to elute the truncated sequences without the DMT group. Next, the cartridge was treated twice with 1 mL of 2% aqueous trifluoroacetic acid (TFA) solution to remove the DMT group followed by 1 mL of deionized water (twice). Then, 1 mL of 50% acetonitrile/water solution was used to elute the desired full-length oligonucleotide that was collected,
- 20 quantified and analyzed.

EXAMPLE 2: Stability study of ApTOLL and ApSION 1-6

- The objective of this study was to determine the stability of the aptamers ApTOLL, ApSION 1,
- 25 ApSION 2, ApSION 3, ApSION 4, ApSION 5 and ApSION 6 against nucleases (λ -exonuclease and DNase I), and in human, rat and non-human primate (NHP) plasma.

2.1 Materials and methods

2.1.1 Materials

Nucleases λ -exonuclease (New England Biolabs) and DNase I (Thermo Scientific) and human plasmas were provided by UCS-Aptamers. ApTOLL, ApSION 1, ApSION 2, ApSION 3, ApSION 4, ApSION 5 and ApSION 6 aptamers and rat and NHP plasmas were provided by Aptatargets.

2.1.2 Stability against nucleases (λ -exonuclease and DNase I)

Aptamers (at a concentration of 1.2 μ M), previously structured in PBS+ 1mM Mg₂Cl, were incubated with 0, 0.01, 0.1 or 1 unit of λ -exonuclease or DNase I for 5 minutes at 37 °C. The enzymes were inactivated at 65 °C for 10 min in the presence of 0.1 mM EDTA. Samples were run on at 3% concentration MS-8 Agarose gel (Conda) in TAE buffer and visualized with GelRed (Biotium). PUC19 DNA/MspI (HpaII) Marker (Thermo Scientific™, Ref. SM0222) was used as molecular weight marker. The bands were quantified with the Biorad "Image Lab" program. The results are expressed as % of aptamer with respect to samples incubated in the absence of nuclease and are represented as the mean $\pm$ S.E.M. For statistical analysis, a one-sample t-test was used.

2.1.3 Stability in plasma

Aptamers (at a concentration of 4 μ M), previously structured in PBS+ 1mM MgCl₂, were incubated with human, rat or non-human primate (NHP) plasma for 0, 24, 48 or 72 hours at 37 °C. Plasma nucleases were inactivated at 65°C for 10 minutes and samples were frozen at -20 °C until the end of the assay. All assays were repeated by incubating the samples with 1.8 μ g/mL proteinase K for 15 min at 55 °C, to degrade plasma proteins and improve the resolution of agarose gels. The amount of aptamer was quantified as in section 2.1.2. The results are expressed as % aptamer with respect to time 0 and are represented as the mean $\pm$ S.E.M. For statistical analysis a one-sample t-test was used.

2.2 Results

2.2.1 Stability against nucleases (λ -exonuclease and DNase I)

All aptamers were resistant to exonuclease at all concentrations tested (FIG. 1(A)). The aptamer ApTOLL, unmodified, was completely degraded in the presence of 1 unit of DNase I and more than 40% in the presence of 0.1 unit. All other aptamers were resistant to DNase I except ApSION 6 which was degraded by approximately 40% in the presence of 0.1-1 unit of nuclease (FIG. 1(B)).

2.2.2 Stability in plasma

All aptamers were resistant to the nucleases present in human plasma. Also, aptamers were resistant to nuclease in rat plasma, except ApTOLL and ApSION 3 which were degraded by approximately 20% and 35%, respectively, after 24 hours of incubation with rat plasma. The values at 48 and 72 hours were similar to that showed at 24 hours. In the case of stability of the aptamers in the presence of NHP plasma, it was observed that, although the various aptamers, except ApTOLL, ApSION 1

and ApSION 5, degraded by approximately 20%, only ApSION 3 degraded in an incubation time-dependent manner, with degradation reaching 40% after 72 hours. See results in FIG. 2.

Accordingly, it can be concluded that the nuclease stability (DNase I) of the different ApSIONs was better than that shown by ApTOLL with the exception of ApSION 6, which was degraded by 40%. Further, all aptamers were resistant to the nucleases present in the human plasmas used in this study, and ApSION 3 was the ApSION most sensitive to the nucleases present in rat and NHP plasmas.

EXAMPLE 3: Plasma protein binding study of human, rat and NHP plasma

The objective of this study was to quantify the amount of ApSION 2 and ApSION 4 that bind to human, rat and non-human primate plasma proteins.

3.1 Materials and methods

FITC-conjugated ApSION 2 and ApSION 4 aptamers and rat and NHP plasmas were provided by AptaTargets, S.L. Human plasmas were provided by UCS-Aptamers.

FITC-ApSION 2 and FITC-ApSION 4 aptamers (200 pmoles) structured in 100 µl of PBS + 1 mM MgCl₂ were incubated with 150 µl of human, rat or non-human primate (NHC) plasma for 30 min at 37 °C. The samples were adjusted to a final volume of 500 µl with PBS + MgCl₂, and loaded onto a HiPrep 16/60 Sephacryl S-200 High Resolution gel filtration column (GE Healthcare) and ÄKTA prime plus system (GE Healthcare). Fractions of 1.5 mL were collected and 100 µl of each fraction was pipetted into a p96 plate to measure the fluorescence from each TECAN Infinity spectrophotometer well ($\lambda_{\text{excitation}}$ 490 nm and $\lambda_{\text{emission}}$ 525 nm).

3.2 Results

The column was calibrated using the following standards: cytochrome C, albumin and aldolase, and dextran blue to calculate the exclusion volume (Vo). Elution volume (Ve) data were obtained for each of the standard proteins, and partition coefficients (Kav) were calculated (Table 2).

Table 2. Sephacryl S-200 Column Calibration.

	PM (kDa)	Ve (mL)	Kav
Cytochrome C	12.4	78.8	0.5
Albumin	67	53.25	0.19
Aldolase	158	47.32	0.12
Dextran blue	2000	37.1 (Vo)	

* $K_{av} = \frac{V_e - V_o}{V_t - V_o}$. The semilogarithmic representation of the molecular weights of the standard proteins versus their respective Kav provides the calibration line.

After column calibration, the elution volume of the aptamers (200 pmoles) and the fluorescence intensity of each fraction were determined. The binding of each aptamer to three human, rat and non-human primate plasmas was analyzed as described in the methodology (FIG. 3). To determine the percentage of aptamer bound to plasma proteins, the following formula was used:

5

$$\frac{\sum \text{Fractions 35-54}}{\sum \text{All fractions}} \times 100$$

The fractions comprised between 35 (beginning of elution of the proteins excluded from the column) to 54, the volume at which the aptamer begins to elute when injected only to the column. The percentage obtained for each sample was subtracted from the percentage obtained when the aptamer alone was loaded onto the column. Table 3 shows the results corresponding to the quantification of the aptamer bound to plasma proteins, expressed in percentage of bound aptamer.

10

Table 3. Amount of aptamer bound to plasma proteins.

	ApSION 2	Media ± SEM	ApSION 4	Media ± SEM
Human 1	3.2	4.17±1.88	0	2.2±1.81
Human 2	1.5		0.8	
Human 3	7.8		5.8	
Rat 1	6.4	5.5±1.27	7.6	4.1±1.75
Rat 2	3.0		2.0	
Rat 3	7.1		2.8	
NHP 1	6.3	3.5±1.44	2.4	3.3±2.14
NHP 2	1.5		7.4	
NHP 3	2.7		0.15	

15

The amount of aptamer binding to plasma proteins did not exceed 10% in any of the species analyzed, with percentages similar to those obtained with the unmodified ApTOLL aptamer. Therefore, the differences in binding to plasma proteins between the two aptamers was not significant.

20 **EXAMPLE 4: Aptamer specificity study against TLRs**

The purpose was to determine the antagonist activity of ApSION 1-6 for TLR-2, TLR-4 and TLR-5 receptors.

25 4.1 Materials and methods

Protein alkaline phosphatase (SEAP) assay was used to determine SEAP activity by colorimetric detection in the cell supernatant of HEK-Blue™ hTLR2/4/5 cells. The SEAP assay protocols HEK-Blue™ hTLR2 cells (Invivogen, ref. Hkb-htlr2), HEK-Blue™ hTLR4 cells (Invivogen, ref. Hkb-htlr4) and HEK-Blue™ hTLR5 cells (Invivogen, ref. Hkb-htlr5) were used, respectively. If the activity of the

SEAP enzyme is high, the aptamer will have agonist activity, and if this activity decreases with respect to the control, the aptamer will have antagonist capacity.

To detect the antagonist activity of the aptamers against TLR-2, TLR-2 receptor of HB-hTLR2 cells was stimulated with its specific ligand Pam3 (10 ng/ml) 1 hour before adding the aptamer. For the antagonist activity of the aptamers against TLR-4, TLR-4 receptor of HB-hTLR4 cells was stimulated with its specific ligand LPS-Ek up (1 ng/ml) 1 hour before adding the aptamer. For the antagonist activity of the aptamers against TLR-5, TLR-5 receptor of HB-hTLR5 cells was stimulated with its specific ligand FLA-ST up (10 ng/ml) 1 hour before adding the aptamer.

For this assay, 1x10⁴ cells/well were seeded on p96 plates in sextuplicate, and after 24 h the aptamers were added at 20 nM and 200 nM. At the same time, the specific agonist for each TLR was used as a control measure of receptor activity as mentioned in the previous paragraph. The SEAP activity was measured 18 h after aptamer addition, by adding QUANTI-Blue™ Solution (Invivogen, ref. rep-qbs). The absorbance was measured at 620 nm after half an hour of incubation at 37 °C in the Infinite F200 instrument. The results were expressed as percentage of activity with respect to the control.

4.2 Results

The antagonist activity of all aptamers against TLR-2, TLR-4 and TLR-5 is shown in FIG. 4 and in Table 4 and Table 5 below. All aptamers ApSION 1-6 had TLR-4 antagonist activity, but not TLR-5 antagonist activity. Further, ApSION 1 and ApSION 3 had TLR-2 antagonist activity.

Table 4. Percentage of activity with respect to the control.

	TLR-2		TLR-4		TLR-5	
	Mean%	error	Mean%	error	Mean%	error
Agonist control	100	0.1	100	0.5	100	0.5
ApTOLL 20 nM	100	0	86.2	0.2	103.3	0.4
ApTOLL 200 nM	103.8	1	81.1	2.4	93.6	1.7
ApSION 1 20 nM	83.1	2.9	64.8	3.8	98.2	1.8
ApSION 1 200 nM	58.1	4.1	64	3.7	93.9	1.8
ApSION 2 20 nM	101	0.9	71.7	2	93.3	1.4
ApSION 2 200 nM	100.8	0.4	59	3.2	98.2	1.9
ApSION 3 20 nM	86.7	0	65.7	0	104	0
ApSION 3 200nM	65.4	0	72.9	0	104.2	0
ApSION 4 20 nM	98.1	7.9	74.6	5.4	95.6	1.6
ApSION 4 200nM	95.8	9.7	66.8	4.4	98.5	1.3
ApSION 5 20 nM	110.1	3.7	77.4	1.9	98.8	1.8
ApSION 5 200 nM	106.2	5.9	69.9	5.7	101.5	1.5
ApSION 6 20 nM	104.1	0	86.1	0	96.5	0
ApSION 6 200 nM	88.4	0	61.4	0	99.5	0

Table 5. Antagonist activity against TLR-2, TLR-4 and TLR-5 receptors.

	TLR-2 receptor	TLR-4 receptor	TLR-5 receptor
ApSION 1	YES	YES	NO
ApSION 2	NO	YES	NO
ApSION 3	YES	YES*	NO
ApSION 4	NO	YES	NO
ApSION 5	NO	YES	NO

*Not concentration dependent.

STUDY OF THE EFFECT OF APTOLL AND APSION 1-6 ON BREAST CANCER INVASION

EXAMPLE 5: Study of the expression of TLR-4 in triple negative breast tumor lines

High TLR-4 expression can be associated with metastasis and poor prognosis in several types of cancer, including breast cancer. Therefore, the aim of this study was to measure the expression of TLR-4 in triple negative breast tumor lines.

5.1 Materials and methods

TLR-4 expression in MDA-MB-231 and SUM159 tumor lines was assessed through TLR-4 mRNA levels. For these assays, HEK-293T cells, non-tumorigenic cells derived from human embryonic kidney cells that do not overexpress TLR-4 protein, were used as a negative control.

5.1.1 Cell cultures

MDA-MB-231 tumor line was maintained in adherence-grown cell cultures in 75 cm² flasks with DMEM:HAM'S F12 medium (Biowest SAS, France) (1:1) supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 25 µg/ml amphotericin, at 37 °C in an incubator with 5% CO₂ and 95% O₂.

SUM159 tumor line was maintained in adherent cell cultures, in 75 cm² flasks with HAM'SF12 medium (Biowest SAS, France), supplemented with 5% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 25 µg/ml amphotericin, 5 mg/ml insulin, and 1 µg/ml hydrocortisone, at 37 °C in an incubator with 5% CO₂ and 95% O₂.

5.1.2 mRNA analysis

To obtain RNA, cells were collected by lifting with trypsin and centrifuging at 400 g for 5 min. The cell pellet was resuspended in 500 µl of NucleoZOL reagent and the protocol was followed as indicated by the manufacturer.

cDNA was obtained using SensiFAST™ cDNA Synthesis kit (Bioline, UK) as indicated by the manufacturer. Subsequent qPCR was performed on a StepOnePlus Real-Time PCR Systems thermal

cycler with the AceQ SYBR® qPCR Master Mix kit. The oligonucleotide pairs used in this study are listed in Table 6.

Table 6. Oligonucleotides.

	Forward	Reverse
TLR-4	AGACCTGTCCCTGAACCCTAT (SEQ ID NO: 9)	CGATGGACTTCTAAACCAGCCA (SEQ ID NO: 10)
Actin	TGGACTTCGAGCAAGAGATG (SEQ ID NO: 11)	GAAGGAAGGCTGGAAGAGTG (SEQ ID NO: 12)

5.2 Results

The data shown in FIG. 5 confirm that the HEK-293T cell line showed residual TLR-4 mRNA expression, whereas both MDA-MB-231 and SUM159 cells showed significant and similar TLR-4 mRNA expression. Results confirm that MDA-MB-231 and SUM159 breast tumor cells express TLR-4 protein, which allowed to characterize and validate the effect of the ApTOLL aptamer, as well as ApSION 1-6 in breast tumor cells.

EXAMPLE 6: Study of TLR-4 expression in mammospheres of MDA-MB-231 and SUM159

In order to determine if there were differences in the expression levels of the TLR-4 membrane receptor in these tumor lines grown in an adherent manner, or forming mammospheres, the expression of the TLR-4 mRNA was measured using third generation spheres, as well as cells grown in adherence, of these breast tumor lines.

6.1 Materials and methods

MDA-MB-231 and SUM159 tumor lines were maintained forming mammospheres to obtain cancer stem cells (CSCs), in suspension, in 25 cm² ultra-low attachment (ULA) (Corning, USA), low adherent flasks, with the same culture medium: HAM'S12:DMEM (1:1), B27 (1:50), 5 µg/ml insulin, 20 ng/ml EGF, 20 ng/ml bFGF, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 1 µg/ml hydrocortisone, at 37 °C in a 5% CO₂, 95% O₂ incubator.

Both cell types were seeded on p24 plates at a cell density of 50,000 cells/well for the 2D cells and on p24 ULA plates at a cell density of 5,000 cells/well for the 3D cells. Adherent cells were harvested 24h after seeding, and spheres were harvested 15 days after formation, for subsequent RNA extraction to measure TLR-4 mRNA levels by real-time PCR, using β-actin as an endogenous control. The oligonucleotide pairs used in this study are listed in Table 6.

6.2 Results

FIG. 6 shows how TLR-4 mRNA is also overexpressed in mammospheres and slightly higher than in adherent cells. As the generated mammospheres possess high amounts of CSCs with metastatic capacity, it can be concluded that these CSCs also overexpress the TLR-4 membrane receptor.

EXAMPLE 7: Study of the activity of aptamers ApTOLL and ApSION 1-6 on the efficiency of mammosphere formation

- 5 The objective was to determine the effect of the aptamers ApTOLL, ApSION 1, ApSION 2, ApSION 3, ApSION 4, ApSION 5 and ApSION 6 on the sphere-forming ability of breast tumor cells by studying the number, perimeter, and size of spheres, and mTLR-4 levels.

7.1 Materials and Methods**10 7.1.1 Materials**

Cells and all necessary material for sphere growth, RNA extraction kits, qPCR, etc. was provided by UCS-Aptamers. ApTOLL, ApSION 1, ApSION 2, ApSION 3, ApSION 4, ApSION 5 and ApSION 6 aptamers were provided by AptaTargets, S.L.

15 7.1.2 Third generation spheres obtention

- SUM159 or MDA-MB-231 cells were seeded in a P100 plate at a density of $5-6 \times 10^6$ cells. After 24 hours, the cells present in the supernatant, which are the cells capable of forming spheres (stem cells), were collected and seeded in medium defined for spheres (DMEM medium: HAM'S 1:1 supplemented with B27, 5 $\mu\text{g/ml}$ insulin, 1 $\mu\text{g/ml}$ hydrocortisone, 20 ng/ml EGF, 20ng/ml bFGF, 100
20 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin, and 25 $\mu\text{g/mL}$ amphotericin) in a low-adherent culture flask (Corning Ultra-Low Attachment 25 cm^2 flask). They were maintained for 15 days, obtaining the first generation. To obtain the second generation, the spheres were treated with trypsin, centrifuged and the sedimented cells are reseeded in defined medium in a low-adherent culture flask for 15 days.

25 7.1.3 Treatment of cells with aptamers

- Second generation cells were trypsinized, counted and seeded 5000 cells/well in a low adherence p24 plate (Corning Ultra-low binding multiwell plates p24), three wells per experimental spot in the absence or presence of 200 nM of aptamer, previously structured in PBS + 1mM MgCl_2 . The aptamers were added again three times per week for 15 days. After 15 days, the spheres in each well
30 were counted, photos are taken (x10) and the diameter and perimeter were measured with the Fiji program (at least 30 spheres per experimental point). Finally, the medium was collected (the three wells of each experimental point were joined), centrifuged at 1500 rpm, 5 min, and the supernatant was kept to quantify cytokines by ELISA. The cells were trypsinized, washed in 1 ml of medium, centrifuged and total RNA is obtained using NucleoZol (Macherey Nagel).

35

7.1.4 Measurement of TLR-4 mRNA levels

- cDNA was obtained using the cDNA Synthesis kit (Ecogen) and mTLR-4 levels were measured by real-time PCR using the AceQ qPCR SYBR[®] Green Master Mix kit (Vazyme) according to the supplier's instructions, using β -actin as an endogenous control. The oligonucleotide pairs used in this
40 study are listed in Table 6.

7.2 Results

Three assays were performed with ApTOLL and ApSION 1-6 at a concentration of 200 nM. As seen in FIG. 7, there is a tendency for aptamers ApSION 1, 2, 3 and 4 to reduce the number of spheres by 30-50% while all of them except ApSION 6 seem to have an effect on the size of the spheres, both in diameter and perimeter.

Measurement of TLR-4 mRNA levels showed that ApSION 2 and ApSION 4 produced a significant reduction of TLR-4 mRNA levels (FIG. 8) by about 50%, whereas ApSION 1 and ApSION 3 reduced TLR-4 mRNA levels by approximately 20%.

In view of all these results, it was decided to perform two new assays with ApSION 2 and 4 aptamers to compare their inhibitory activity on mammosphere formation with that produced by ApTOLL.

The results obtained (FIG. 9(A)) confirm that treatment with ApTOLL or the modified aptamers ApSION 2 and 4 inhibits the ability of SUM159 cells to form spheres and the size of the spheres formed. These results were confirmed in another triple-negative breast tumor line, MDA-MB-231 (FIG. 9(B)).

TLR-4 levels from the 5 assays performed in SUM159 cells confirm that all three aptamers produce a significant decrease in TLR-4 mRNA levels, which are reduced by about 50% (FIG. 10).

Therefore, it can be concluded that the modified aptamers ApSION 2 and ApSION 4 inhibit the ability of stem cells derived from triple-negative breast tumor cells (SUM159 and MDA-MB-231) to form spheres comparable to that observed with ApTOLL; and also that all three aptamers reduce TLR-4 mRNA levels significantly.

EXPERIMENTS WITH EXPERIMENTAL AUTOIMMUNE NEURITIS (EAN) MOUSE MODEL

EXAMPLE 8: Efficacy study of ApTOLL, ApSION 2 and ApSION 4 in a preclinical model of inflammatory autoimmune neuropathy disorder or Guillain-Barré syndrome (GBS)

The aim of the study was to evaluate the positive efficacy of ApTOLL, ApSION 2 and ApSION 4 in a preclinical GBS model, and to compare the efficacy between the new candidates (ApSION 2 and ApSION 4) and ApTOLL, when administered at a single dose by intravenous route in male mice when the inflammatory neuropathy is established.

8.1 Materials and methods

8.1.1 Compound formulation

Table 7. Compound formulation of ApTOLL, ApSION 2 and ApSION 4

Vehicle	PBS + 1 mM MgCl ₂
Concentration treatment	ApTOLL: 0.91 mg/kg ApSION 2: 0.45 mg/kg ApSION 4: 0.45 mg/kg
Frequency of preparation	Fresh just before administration
Frequency of treatment	Single dose at the indicated time
Administration route	Intravenous
Administration volume	5 mL/kg

8.1.2 Composition of the groups

Table 8. Composition of the six groups

Group number	Group name	GBS induction?	Administered compound	Administration route	Number of administrations	Dose	Administration time point	Number of animals
1	sham	No	-	-	-	-	-	8
2	GBS+vehicle 1	Yes	Vehicle 1	Intravenous	1	-	Day 10	8
3	GBS+positive control	Yes	3,6'- Dithiothaldionide (DTT)	Intraperitoneal	12	50mg/kg	From Day 10 to Day 22	8
4	GBS+ApTOLL	Yes	ApTOLL	Intravenous	1	0.91 mg/kg	Day 10	8
5	GBS+ApSION 2	Yes	ApSION 2	Intravenous	1	0.45 mg/kg	Day 10	8
6	GBS+ApSION 4	Yes	ApSION 4	Intravenous	1	0.45 mg/kg	Day 10	8

5 8.1.3 Animals

Experimental autoimmune neuritis (EAN), induced by the subcutaneous injection of sciatic nerve homogenate combined with Freund's adjuvant, is a widely used animal model of demyelinating peripheral neuropathy that has provided valuable insights into pathogenesis and a means to assess novel therapeutic agents. This model can be induced in different species such as rabbits, rats, mice, and guinea pigs, and all models present chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) around two weeks after the last subcutaneous injections, allowing to study the efficacy of new compounds targeting inflammatory neuropathy in a short time period. The characteristics of the specific animal model performed in this study are detailed in Table 9.

15 Table 9. Characteristics of EAN mouse model

Sex/Strain/Species/age:	Male BL6/C57 Mice 8 weeks old
Approximate weight at initiation of the study:	22.0 g ± 2.5
Animal number:	8 mice par group Total: 48 mice

Number of groups:	Group 1 : Sham control Group 2 : GBS+ vehicle Group 3 : GBS+DTT (positive control) Group 4 : GBS+ApTOLL Group 5 : GBS+ApSION 2 Group 6 : GBS+ApSION 4
Animal identification:	At the beginning of the study, animals were identified with a number on the tail. Each parameter was noted in the lab book.
Experimental <i>in vivo</i> time duration	22 days: Readouts measures at day 1 and day 22 Sampling at day 22 Model induction by subcutaneous injections at day 2, day 5 and day 9 Compound administration at day 10

Animal protocol was approved by the Animal Studies Committee of Languedoc Roussillon. Animal health, mortality and clinical signs were examined every day to ensure that only animals in good health enter to the testing procedures and follow up the study.

5

8.1.4 Sciatic nerve homogenate injection for model induction

Sciatic nerves were sampled from BL6/C57 mice. Nerve was weighted, cut in small parts and the Freund's complete adjuvant was added. Study mice were treated by subcutaneous injection of the sciatic nerve homogenate + Freund's adjuvant mixture at 10 mL/kg. For each treatment, 3 different body sites injections were performed. Control mice also received three subcutaneous injections of PBS + Freund's adjuvant at the same 3 different body sites.

10

8.1.5 Sciatic nerve electrophysiology

Standard electromyography was performed on mice anesthetized with ketamine/xylazine mixture, by placing electrodes along the nerve at the sciatic notch (proximal stimulation) and along the tibial nerve above the ankle (distal stimulation). Supramaximal square-wave pulses, lasting 10 ms at 1 mA were delivered and compound muscle action potential (CMAP) was recorded from the intrinsic foot muscles using steel electrodes. Both amplitudes and latencies of CMAP were determined. The distance between the 2 sites of stimulation was measured alongside the skin surface with fully extended legs, and nerve conduction velocities (NCVs) were calculated automatically from sciatic nerve latency measurements.

15

20

8.1.6 Grip strength test

Neuromuscular strengths of all mice were assessed in standardized grip strength tests for hind limbs. Hind limb grip strength was measured by supporting the limbs on a horizontal T-bar connected to a gauge and pulling the animal's tail. The maximum force (measured in newtons) exerted on the T-bar before the animal lost its grip was recorded, and the mean of 3 repeated measurements was calculated. Finally, the data were averaged for each treated group.

25

8.1.7 Rotarod

A rotating rod apparatus was used to measure walking performances, coordination and balance. Mice were first given a 1-days pretraining trial (test learning) to familiarize them with the rotating rod. Latency to fall was measured at a successively increased speed from 4 to 40 rpm over a 300-second max. time period. Each animal underwent 3 trials a day. For each day, values from the 3 trials were averaged for each animal, and then were averaged for each group.

8.1.8 Plasma sampling, sciatic nerve sampling and biomarker quantification by ELISA

Blood collection from the tail vein is the blood collection technique that allows for maximum allowable sample volume with minimal trauma to the animal. The tail vein was punctured and blood was collected directly into a 1 mL syringe previously coated with anticoagulant heparin. Around 100 µl of blood was sampled in a heparinized microtube and stored at -20 °C before ELISA analysis.

After blood sampling, mice were sacrificed by cervical dislocation. Then, the left leg skin was opened, and the full sciatic nerve was sampled. Each sciatic nerve was fixed in glutaraldehyde 2.5% and PFA 4% overnight at 4 °C and stored in PBS buffer at 4 °C.

For plasma IL-6 and TNF- α quantification, plasma was diluted at 1/10 using 10 μ l of each sample + 90 μ l of sterile PBS. Samples were analyzed by duplicated using ELISA colorimetric method at 450 nm using 100 μ l per well.

8.1.9 In vivo study phase

FIG. 11 shows the study scheme, and Table 10 the study flowchart. All procedures at each experimental time are detailed below.

Table 10. Study flowchart. D: days, e.g., D-6 is day -6.

[illegible]

8.1.10 Statistical analysis

Descriptive statistics by groups were expressed as mean $\pm$ SEM for continuous variables. Statistical significances were determined using 2-way ANOVA, followed by a Bonferroni multiple comparisons post hoc test, allowing comparisons between groups, assuming the normal distribution of the variable and the variance homoscedasticity. Statistical analyses were performed using GraphPad Prism version 5.02 for Windows, GraphPad Software, La Jolla California USA. A P value of less than 0.05 was considered significant.

8.2 Results

8.2.1 Mortality and body weight

No mortality was observed during the study. No pathological clinical sign was observed during the study.

As expected, significant decrease of the body weight was observed in the GBS + vehicle group compared to the sham (negative control) group.

Even if slight increases of body weights were observed in the groups treated with DTT, ApTOLL and ApSION 2, these increases were not statistically significant compared to the vehicle treated group at the analyzed time point suggesting a partial efficacy of the compounds on the body weight at these experimental conditions.

However, significant increase of body weight was observed in the animals treated with ApSION 4 compared to the vehicle treated group from day 13 (FIG. 12) showing a positive effect of this compound on the body mass at these experimental conditions.

8.2.2 Rotarod

Similar rotarod latencies were observed between the six groups at the baseline (day 1). As expected, neuromotor and coordination impairment was observed at day 22 in the GBS + vehicle treated group compared to the sham (negative control) group, characterized by a significant decrease of the rotarod latency, showing the presence of the inflammatory neuropathy onset.

Even if slight increase of rotarod latency was observed in the groups treated with DTT and ApTOLL at day 22, this increase was not statistically significant compared to the vehicle treated group probably due to the number of animals included in the group (FIG. 13) and suggesting the absence of significant efficacy of the compounds at these experimental conditions.

Importantly, significant increase of rotarod latency was observed in the animals treated with ApSION 2 and ApSION 4 compared to the vehicle treated group at day 22 (FIG. 13), showing positive efficacy of both compounds on the neuromotor impairment induced by the inflammatory neuropathy.

8.2.3 Grip test

Similar grip strengths were observed between the six groups at the baseline (day 1). As expected, neuromuscular impairment was observed at day 22 in the GBS + vehicle treated group compared to the sham (negative control) group, characterized by a significant decrease of the grip strength, confirming the presence of the inflammatory neuropathy onset.

Significant increase of the grip strength was observed in the animals treated with DTT, ApTOLL, ApSION 2 and ApSION 4 compared to the vehicle treated group at day 22 (FIG. 14), showing positive efficacy of the compounds on the neuromuscular impairment induced by the inflammatory neuropathy.

8.2.4 Sciatic nerve electrophysiology

8.2.4.1 Compound muscle action potential (CMAP) amplitude

Similar CMAPs were observed between the six groups at the baseline (day 1). As expected, nerve conduction impairment was observed at day 22 in the GBS+vehicle treated group compared to the sham group, characterized by a significant decrease of the CMAP amplitude.

No statistical differences were observed in the CMAP amplitude of animals treated with DTT and ApTOLL compared to the GBS + vehicle group at day 22 (FIG. 15) suggesting the absence of efficacy at these experimental conditions.

Importantly, significant increase of CMAP amplitude was also observed in the animals treated with ApSION 2 and ApSION 4 compared to the GBS + vehicle group at day 22 (FIG. 15), showing positive efficacy of both compounds on the electrophysiology impairment induced by the inflammatory neuropathy.

Because the CMAP amplitude is directly linked to the functionality and integrity of the neurons of the peripheral nerves, these results suggest that ApSION 2 and ApSION 4 compound directly or indirectly target the axonopathy induced by the inflammatory neuropathy at these experimental conditions.

8.2.4.2 Nerve conduction velocity (NCV)

Similar NCVs were observed between the six groups at the baseline (day 1). As expected, nerve conduction impairment was observed at day 22 in the GBS+vehicle treated group compared to the sham group, characterized by a significant decrease of the NCV.

No statistical differences were observed in the NCVs of animals treated with DTT, ApTOLL and ApSION 2 compared to the GBS + vehicle group at day 22 (FIG. 16) suggesting the absence of efficacy at these experimental conditions.

Importantly, significant increase of NCV was also observed in the animals treated with ApSION 4 compared to the GBS + vehicle group at day 22 (FIG. 16), confirming the positive efficacy of this compound on the electrophysiology impairment induced by the inflammatory neuropathy.

- 5 Because the NCV is directly linked to the functionality and integrity of the myelin sheath of the peripheral nerves, these results also suggest that the compound ApSION 4 directly or indirectly targets myelinating Schwann of the peripheral nervous system at these experimental conditions.

8.2.5 Plasma TNF- α quantification

- 10 Plasma cytokine TNF- α biomarker was quantified by ELISA method to determine the evolution of the inflammation and the efficacy of the compounds on the neuropathy induced by the inflammation. As expected, significant increase of the plasma TNF- α concentration was observed in the GBS+vehicle treated group compared to the sham (negative control) group at day 22.
- 15 Significant decrease of plasma TNF- α concentration was observed in the animals treated with DTT, ApTOLL, ApSION2 and ApSION4 at day 22 (FIG. 17), showing a positive efficacy of the compounds on the inflammation from a biomarker point of view.

8.2.6 Plasma IL-6 quantification

- 20 Plasma cytokine IL-6 biomarker was also quantified by ELISA method to confirm the evolution of the inflammation and the efficacy of the compounds on the neuropathy induced by the inflammation. As expected, significant increase of the plasma IL-6 concentration was observed in the GBS+vehicle treated group compared to the sham (negative control) group at day 22.
- 25 Significant decrease of plasma IL-6 concentration was observed in the animals treated with DTT, ApTOLL, ApSION2 and ApSION4 at day 22 (FIG. 18), confirming the positive efficacy of the compounds on the inflammation from a biomarker point of view.

8.3 Conclusion

- 30 As expected, neuromuscular and neuromotor impairment, decrease of nerve conduction amplitude and velocity, increase of plasma inflammatory cytokines (IL-6 & TNF- α) was observed in the preclinical GBS + vehicle treated group compared to the sham (negative control) group at day 22.

- Even if slight increase of neuromotor, neuromuscular and electrophysiological performances was observed in the groups treated with DTT and ApTOLL at day 22, this increase was no statistically significant compared to the vehicle treated group in some tests probably due to the number of animals included in the group. However, significant decrease of plasma TNF- α and IL-6 concentrations was observed in the animals treated with DTT and ApTOLL at day 22 suggesting the positive efficacy of both compounds on the inflammation from a biomarker point of view.

Importantly, significant increase of neuromotor, neuromuscular and electrophysiological performances was observed in the groups treated with ApSION 2 and ApSION 4 compared to the GBS + vehicle group at day 22, confirming the positive efficacy of both compounds on the electrophysiology impairment induced by the inflammatory neuropathy. Significant decrease of plasma TNF- α and IL-6 concentrations was also observed in ApSION 2 and ApSION 4 treated animals confirming these positive data from a biomarker point of view.

EXAMPLE 9: Dose-response efficacy study of ApSION2 and ApSION 4 in a preclinical mouse model of inflammatory autoimmune neuropathy disorder or Guillain-Barré syndrome (GBS)

The aim of the study was to evaluate the efficacy of ApSION 2 and ApSION 4 in a preclinical Guillain-Barré syndrome (GBS) model when administered at five different doses by intravenous route in male mice when the inflammatory neuropathy is established.

9.1 Materials and methods

9.1.1 Compound formulation

Table 11. Compound formulation of ApSION 2 and ApSION 4

Vehicle	PBS + 1 mM MgCl ₂
Concentration treatment	ApSION 2: 0.23mg/kg, 0.45mg/kg, 0.9mg/kg, 1.8mg/kg & 3.6mg/kg ApSION 4: 0.23mg/kg, 0.45mg/kg, 0.9mg/kg, 1.8mg/kg & 3.6mg/kg
Frequency of preparation	Fresh just before administration
Frequency of treatment	Single dose at the indicated time
Administration route	Intravenous
Administration volume	5 mL/kg

9.1.2 Composition of the groups

Table 12. Composition of the eleven groups

Group number	Group name	GBS induction?	Administred compound	Administration route	Number of administrations	Dose	Administration time point	Number of animals
1	GBS+vehicle 1	Yes	Vehicle 1	Intravenous	1	-	Day 10	10
2	GBS+ApSION 2 0.23mg/kg	Yes	ApSION 2	Intravenous	1	0.23 mg/kg	Day 10	10
3	GBS+ApSION 2 0.45mg/kg	Yes	ApSION 2	Intravenous	1	0.45 mg/kg	Day 10	10
4	GBS+ApSION 2 0.9mg/kg	Yes	ApSION 2	Intravenous	1	0.91 mg/kg	Day 10	10
5	GBS+ApSION 2 1.8mg/kg	Yes	ApSION 2	Intravenous	1	1.8 mg/kg	Day 10	10
6	GBS+ApSION 2 3.6mg/kg	Yes	ApSION 2	Intravenous	1	3.6 mg/kg	Day 10	10
7	GBS+ApSION 4 0.23mg/kg	Yes	ApSION 4	Intravenous	1	0.23 mg/kg	Day 10	10
8	GBS+ApSION 4 0.45mg/kg	Yes	ApSION 4	Intravenous	1	0.45 mg/kg	Day 10	10
9	GBS+ApSION 4 0.9mg/kg	Yes	ApSION 4	Intravenous	1	0.91 mg/kg	Day 10	10
10	GBS+ApSION 4 1.8mg/kg	Yes	ApSION 4	Intravenous	1	1.8 mg/kg	Day 10	10
11	GBS+ApSION 4 3.6mg/kg	Yes	ApSION 4	Intravenous	1	3.6 mg/kg	Day 10	10

9.1.3 Animals

EAN was induced as indicated in EXAMPLE 8.1.3. The characteristics of the specific animal model performed in this study are detailed in Table 13.

5

Table 13. Characteristics of EAN mouse model

Sex/Strain/Species/age:	Male BL6/C57 Mice 8 weeks old
Approximate weight at initiation of the study:	22.0 g ± 2.5
Animal number:	10 mice par group Total: 110 mice
Number of groups:	Eleven groups Group 1: GBS+ vehicle Group 2: GBS+ApSION 2 0.23mg/kg Group 3: GBS+ApSION 2 0.45mg/kg Group 4: GBS+ApSION 2 0.9mg/kg Group 5: GBS+ApSION 2 1.8mg/kg Group 6: GBS+ApSION 2 3.6mg/kg Group 7: GBS+ApSION 4 0.23mg/kg Group 8: GBS+ApSION 4 0.45mg/kg Group 9: GBS+ApSION 4 0.9mg/kg Group 10: GBS+ApSION 4 1.8mg/kg Group 11: GBS+ApSION 4 3.6mg/kg
Animal identification:	At the beginning of the study, animals were identified with a number on the tail. Each parameter was noted in the lab book.
Experimental <i>in vivo</i> time duration	22 days: Readouts measures at day 1 and day 22 Sampling at day 22 Guillain-Barré BBB scoring at day 10, day 16 and day 22 Model induction by subcutaneous injections at day 2, day 5 and day 9 Compound administration at day 10

Animal protocol was followed as in EXAMPLE 8.1.3.

10 9.1.3 Evaluated parameters

The parameters that were evaluated were: sciatic nerve homogenate injection for model induction; sciatic nerve electrophysiology; grup strength test; rotarod; plasma sampling, sciatic nerve sampling and biomarker quantification by ELISA; Guillain-Barré disability BBB locomotor scoring; in vivo study phase; and statistical analysis. These parameters were analyzed as explained in EXAMPLE 8.1.4-
15 8.1.8 and 8.1.10, except for the Guillain-Barré disability BBB locomotor scoring and in vivo study phase which were analyzed as explained below.

9.1.3.1 Guillain-Barré disability BBB locomotor scoring

Individual locomotor performance and recovery was measured using the standardized BBB score (Basso, Beattie and Bresnahan, 1995) at the corresponding time-point analysis following the parameters listed below. Each individual animal scoring was performed by duplicated by two different laboratory technician to avoid experimental subjectivity at the corresponding time-point analysis.

- 0 - No observable hind limb (HL) movement.
- 1 - Slight movement of one or two joints, usually the hip and/or knee.
- 2 - Extensive movement of one joint or extensive movement of one joint and slight movement of one other joint.
- 3 - Extensive movement of two joints.
- 4 - Slight movement of all three joints of the HL.
- 5 - Slight movement of two joints and extensive movement of the third.
- 6 - Extensive movement of two joints and slight movement of the third.
- 7 - Extensive movement of all three joints of the HL.
- 8 - Sweeping with no weight support or plantar placement of the paw with no weight support.
- 9 - Plantar placement of the paw with weight support in stance only (i.e., when stationary) or occasional, frequent, or consistent weight supported dorsal stepping and no plantar stepping.
- 10 - Occasional weight supported plantar steps, no forelimb (FL)-HL coordination.
- 11 - Frequent to consistent weight supported plantar steps and no FL-HL coordination.
- 12 - Frequent to consistent weight supported plantar steps and occasional FL-HL coordination.
- 13 - Frequent to consistent weight supported plantar steps and frequent FL-HL coordination.
- 14 - Consistent weight supported plantar steps, consistent FL-HL coordination; and predominant paw position during locomotion is rotated (internally or externally) when it makes initial contact with the surface as well as just before it is lifted off at the end of stance or frequent plantar stepping, consistent FL-HL coordination, and occasional dorsal stepping.
- 15 - Consistent plantar stepping and consistent FL-HL coordination; and no toe clearance or occasional toe clearance during forward limb advancement; predominant paw position is parallel to the body at initial contact.
- 16 - Consistent plantar stepping and consistent FL-HL coordination during gait; and toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift off.
- 17 - Consistent plantar stepping and consistent FL-HL coordination during gait; and toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel at initial contact and lift off.
- 18 - Consistent plantar stepping and consistent FL-HL coordination during gait; and toe clearance occurs consistently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift off.
- 19 - Consistent plantar stepping and consistent FL-HL coordination during gait; and toe clearance

occurs consistently during forward limb advancement; predominant paw position is parallel at initial contact and lift off; and tail is down part or all of the time.

20 - Consistent plantar stepping and consistent coordinated gait; consistent toe clearance; predominant paw position is parallel at initial contact and lift off; tail consistently up; and trunk instability.

- 5 21 - Consistent plantar stepping and coordinated gait, consistent toe clearance, predominant paw position is parallel throughout stance, consistent trunk stability, tail consistently up.

9.1.3.2 In vivo study phase

FIG. 11 shows the study scheme and Table 14 the study flowchart.

10

Table 14. Study flowchart. D: days, e.g., D-6 is day -6.

Test/Day	D-6	D0	D1	D2	D5	D9	D10	D16	D21	D22	D29
Animal arrival	X										
Body weight and clinical signs	Once a day										
Behavioral test learning		X							X		
Rotarod			X							X	
Grip test			X							X	
EMG (electrophysiology)			X							X	
Neuromotor disability BBB scoring							X	X			
Sciatic nerve homogenate + Freund's adjuvant treatment				X	X	X					
Single ApSION 2 & ApSION 4 administration							X				
Plasma sampling for biomarker										X	
Animal euthanasia										X	
Sciatic nerve sampling										X	
ELISA results (IL-6 & TNF-α)											X

9.2 Results

9.2.1 Mortality and body weight

- 15 No mortality was observed during the study. No pathological clinical sign was observed during the study.

Even if slight increases of body weights were observed in the groups treated with ApSION 2 0.23 mg/kg and ApSION 4 0.23 mg/kg at day 20, 21 and 22, these increases were no statistically significant compared to the vehicle treated group at the analyzed time points.

20

However, significant increase of body weight was observed in the animals treated with ApSION 2 or ApSION 4 at 0.45 mg/kg, 0.9 mg/kg, 1.8 mg/kg and 3.6 mg/kg compared to the vehicle treated group from day 16 showing a positive effect of these compounds on the body mass at these doses (FIG. 19).

25

9.2.2 BBB motor disability scoring

No significant increase of the BBB score was observed in the groups treated with ApSION 2 0.23 mg/kg and ApSION 4 0.23 mg/kg at day 16 and day 22 compared to the vehicle treated group at the analyzed time points (FIG. 20) suggesting absence of compounds efficacy at these experimental conditions.

5

Significant increase of the BBB score was observed in the animals treated with ApSION 2 or ApSION 4 at 0.45 mg/kg, 0.9 mg/kg, 1.8 mg/kg and 3.6 mg/kg compared to the vehicle treated group at day 22 (FIG. 20) showing a positive efficacy of the compounds at these doses on the motor disability.

10 9.2.3 Rotarod

Similar rotarod latencies were observed between the eleven groups at the baseline (day 1). As expected, neuromotor and coordination impairment was observed at day 22 in the GBS+vehicle treated group, characterized by a decrease of the rotarod latency.

15 Even if slight increase of rotarod latency was observed in the groups treated with ApSION 2 and ApSION 4 at 0.23 mg/kg, this increase was no statistically significant compared to the GBS+vehicle treated group at day 22 (FIG. 21), suggesting the absence of significant efficacy of the compounds at this dose.

20 Importantly, significant dose-response increase of rotarod latency was observed in the animals treated with ApSION 2 and ApSION 4 at 0.45 mg/kg, 0.9 mg/kg, 1.8 mg/kg and 3.6 mg/kg compared to the vehicle treated group at day 22 (FIG. 21), showing positive dose-response efficacy of both compounds on the neuromotor impairment induced by the inflammatory neuropathy.

25 9.2.4 Grip test

Similar grip strengths were observed between the eleven groups at the baseline (day 1). As expected, neuromuscular impairment was observed at day 22 in the GBS + vehicle treated group, characterized by a decrease of the grip strength.

30 Even if slight increase of grip strength was observed in the group treated with ApSION 2 0.23 mg/kg, this increase was no statistically significant compared to the GBS+vehicle treated group at day 22 (FIG. 22), suggesting the absence of significant efficacy of the compounds at this dose.

35 Importantly, significant dose-response increase of grip strength was observed in the animals treated with ApSION 2 at 0.45 mg/kg, 0.9 mg/kg, 1.8 mg/kg and 3.6mg/kg and with ApSION 4 at 0.23 mg/kg, 0.45 mg/kg, 0.9 mg/kg, 1.8 mg/kg and 3.6 mg/kg compared to the vehicle treated group at day 22 (FIG. 22), showing positive dose-response efficacy of both compounds on the neuromuscular impairment induced by the inflammatory neuropathy.

40 9.2.5 Sciatic nerve electrophysiology

9.2.5.1 Compound muscle action potential (CMAP) amplitude

Similar CMAPs were observed between the eleven groups at the baseline (day 1). As expected, nerve conduction impairment was observed at day 22 in the GBS+vehicle treated group, characterized by a significant decrease of the CMAP amplitude.

5

Even if slight increase of CMAP amplitude was observed in the groups treated with ApSION 2 and ApSION 4 at 0.23 mg/kg, this increase was no statistically significant compared to the GBS+vehicle treated group at day 22 (FIG. 23), suggesting the absence of significant efficacy of the compounds at this dose.

10

Importantly, significant dose-response increase of CMAP amplitude was observed in the animals treated with ApSION 2 and ApSION 4 at 0.45 mg/kg, 0.9 mg/kg, 1.8 mg/kg and 3.6 mg/kg compared to the vehicle treated group at day 22 (FIG. 23), showing positive dose-response efficacy of both compounds on the nerve conduction impairment induced by the inflammatory neuropathy.

15

9.2.5.2 Nerve conduction velocity (NCV)

Similar NCVs were observed between the eleven groups at the baseline (day 1). As expected, nerve conduction impairment was observed at day 22 in the GBS+vehicle treated group, characterized by a significant decrease of the NCV.

20

Even if slight increase of NCV was observed in the groups treated with ApSION 2 and ApSION 4 at 0.23 mg/kg, this increase was no statistically significant compared to the GBS+vehicle treated group at day 22 (FIG. 24), suggesting the absence of significant efficacy of the compounds at this dose.

25

Importantly, significant dose-response increase of NCV was observed in the animals treated with ApSION 2 and ApSION 4 at 0.45 mg/kg, 0.9 mg/kg, 1.8 mg/kg and 3.6 mg/kg compared to the vehicle treated group at day 22 (FIG. 24), confirming positive dose-response efficacy of both compounds on the nerve conduction impairment induced by the inflammatory neuropathy.

30

9.2.6 Plasma TNF- α quantification

Plasma cytokine TNF- α biomarker was quantified by ELISA method to determine the evolution of the inflammation and the efficacy of the compounds on the neuropathy induced by the inflammation.

35

Significant dose-response decrease of plasma TNF- α concentration was observed in the animals treated with ApSION2 and ApSION4 at 0.23 mg/kg, 0.45 mg/kg, 0.9 mg/kg, 1.8 mg/kg and 3.6 mg/kg at at day 22 (FIG. 25), showing a positive dose-response efficacy of the compounds on the inflammation from a biomarker point of view.

9.2.7 Plasma IL-6 quantification

Plasma cytokine IL-6 biomarker was also quantified by ELISA method to confirm the evolution of the inflammation and the efficacy of the compounds on the neuropathy induced by the inflammation.

5 Significant dose-response decrease of plasma IL-6 concentration was observed in the animals treated with ApSION 2 and ApSION 4 at 0.23 mg/kg, 0.45 mg/kg, 0.9 mg/kg, 1.8 mg/kg and 3.6 mg/kg at day 22 (FIG. 26), confirming a positive dose-response efficacy of the compounds on the inflammation from a biomarker point of view.

9.3 Conclusion

10 As expected, neuromuscular, neuromotor and electrophysiology impairment was observed in the preclinical GBS + vehicle treated group at day 22.

Even if slight increase of neuromotor and electrophysiological performances was observed in the groups treated with ApSION 2 and ApSION 4 at 0.23 mg/kg at day 22, this increase was no statistically significant compared to the vehicle treated group. However, slight but significant decrease of plasma TNF- α and IL-6 concentrations was observed in the animals treated with ApSION 2 and ApSION 4 at 0.23 mg/kg suggesting the partial efficacy of both compounds at this dose from a biomarker point of view.

20 Importantly, significant dose-response increase of neuromotor, neuromuscular and electrophysiological performances was observed in the groups treated with ApSION 2 and ApSION 4 at 0.45 mg/kg, 0.9 mg/kg, 1.8 mg/kg and 3.6 mg/kg compared to the GBS + vehicle group at day 22, confirming the positive dose-response efficacy of both compounds on the neuropathy induced by the inflammatory GBS. Finally, significant dose-response decrease of plasma TNF- α and IL-6 concentrations was also observed in the ApSION 2 and ApSION 4 treated animals at 0.45 mg/kg, 0.9 mg/kg, 1.8 mg/kg and 3.6 mg/kg confirming the positive dose-response efficacy of the compound on these cytokines from a biomarker point of view.

ApTOLL VARIANTS

30

EXAMPLE 10: Mutational analysis of ApTOLL

The effects of percentage of sequence identity and extension at the 5' end and 3' end of ApTOLL sequence (SEQ ID NO: 7) were tested experimentally.

35

First, six variants of ApTOLL sequence presented in Table 15 were obtained and tested. The variants range in sequence identity to SEQ ID NO: 7 between 89.83% and 81.53% and include mutations with respect to the sequence of the aptamer of SEQ ID NO: 7 such as additions at the 5' end of the sequence, additions both at the 5' end and 3' end of the sequence, internal deletions, conservative substitutions, and non-conservative substitutions.

40

Table 15. Mutants (Mut) of ApTOLL sequence. The mutations with respect to ApTOLL sequence are indicated by boldface.

Aptamer	Sequence	% identity to SEQ ID NO: 1	Nucleotides number
ApTOLL (SEQ ID NO: 7)	GGTGTGCCAATAAACCATATCGCCGC GTTAGCATGTACTCGGTGGCCCTAAA TACGAG	100.00	59
ApTOLL-Mut 1 (SEQ ID NO: 13)	CCT GTGCCAATAAA GG ATACCGCCGC GTTAGCATGTACTCGGTGGCCCTAAA TACGAG	91.53	59
ApTOLL-Mut 2 (SEQ ID NO: 14)	GGTGTGCCAATAAACCATATCGCCGC GTTAGCATGTAC AG GTTGGCCCCAA ATAC CT G	91.53	59
ApTOLL-Mut 3 (SEQ ID NO: 15)	TCCT GTGCCAATAAA GG ATACCGCCG CGTTAGCATGTACTCGGTGGCCCTAA ATACGAG	90.76	60
ApTOLL-Mut 4 (SEQ ID NO: 16)	CCT GTGCCAATAAA GG ATACCGCCG CGTTAGCATGTACTCGGTGGCCC-A AATACGAG	90.60	58
ApTOLL-Mut 5 (SEQ ID NO: 17)	TCCT GTGCCAATAAA GG ATACCGCCG CGTTAGCATGTACTCGGTGGCCCTAA ATACGAGT	90.00	61
ApTOLL-Mut 6 (SEQ ID NO: 18)	GGTGTGCCAATAAACCATACCGCCG CGTTAGCATGTAC CT GTTGGCCCCA AATAC AGG	89.83	59

5 The antagonist activity against TLR4 was assessed with Secreted Embryonic Alkaline Phosphatase (SEAP) assay protocol in HEK-Blue hTLR4 cells (InvivoGen, Cat. code Hkb-htlr4). LPS-EK up (InvivoGen Cat. code tlr-peklps) is the TLR4 agonist control. The HEK-Blue hTLR4 cells were obtained by co-transfection of the human TLR4, MD-2 and CD14 co-receptor genes, and an inducible SEAP reporter gene into HEK293 cells. The SEAP reporter gene was placed under the control of an IL-12

10 p40 minimal promoter fused to five NF-κB and AP-1-binding sites. Stimulation with a TLR4 ligand activates NF-κB and AP-1, which induces the production of SEAP. The seeded HEK-Blue hTLR4 cells in a P96 plate have their TLR4 receptor activated with the addition of LPS-EK up. One hour later, the aptamers were added in two final concentrations (20nM, 200nM) and the quantification of reporter protein SEAP expression was made after 16-20 hours.

15

FIG. 27 shows the activity of SEAP reporter protein produced by the TLR4 receptor activation. The natural agonist ligand of TLR4 is LPS (bacterial lipopolysaccharide); in this assay LPS-EK up was

used. The agonist control bar on the left represents that the TLR4 receptor is 100% activated with the mentioned agonist. When the aptamers (control ApTOLL and ApTOLL-Mut 1-6) are added to the activated cells, the TLR4 activity drops and the aptamers bars become shorter than the agonist control bar. Consequently, the aptamers have concentration dependent TLR4 antagonist activity, except for ApTOLL-Mut 4, which is not concentration dependent. The tested mutants have similar behavior, and therefore, FIG. 27 shows that all the ApTOLL variants (ApTOLL-Mut, SEQ ID NO: 13-18) tested had TLR4 antagonist activity comparable to that of ApTOLL aptamer (SEQ ID NO: 7).

Furthermore, a competition assay for TLR4 receptor for these aptamers was also assessed. ELONA-cell assay was made using ApTOLL aptamer (SEQ ID NO: 7) which was modified with a digoxigenin tag (ApTOLL-Dig) and HEK-Blue hTLR4 cells. Equimolecular mixes containing ApTOLL-Dig (100nM) and each ApTOLL-Mut 1-6 (100nM), respectively, were used to identify the possible reduction of digoxigenin related signal in respect to the positive ApTOLL-Dig control (100nM). The positive ApTOLL-Dig control and each mix ApTOLL-Dig/ApTOLL-Mut were added into wells and incubated at 37°C in CO₂ incubator for 15 minutes; then, the wells with cells were washed with PBS, anti-digoxigenin antibody was added, and finally ApTOLL-Dig was detected using ABTS protocol (Roche ref. 11684302001).

FIG. 28 depicts the results of the competition assay, which show that the binding percentage of ApTOLL control aptamer (SEQ ID NO: 7) to TLR4 receptor is 100% when it is added to the cell. Contrarily, when the ApTOLL-Mut aptamers (1-6) in equimolecular mix are respectively added to the cell, the binding percentage bar of ApTOLL aptamer is decreased, thus implying that the ApTOLL-Mut aptamers (1-6) are competing for the same TLR4 receptor binding site than the ApTOLL control aptamer. The ApTOLL-Mut 6 is the aptamer that obtained higher binding affinity to TLR4 receptor (35.9%), and ApTOLL-Mut 2 the lowest. ApTOLL-Mut 1 and ApTOLL-Mut 4 obtained a similar binding percentage (20% and 21%, respectively). Therefore, experimental data presented in FIG. 28 shows that the ApTOLL-Mut aptamers (SEQ ID NO: 13-18) competed with ApTOLL (SEQ ID NO: 7), which would indicate that they are structurally similar and therefore bind to the same binding site on TLR4 as the original ApTOLL aptamer.

In addition, the positions of the mutations (e.g., substitutions, additions) in the secondary structure of the ApTOLL sequence were analyzed (see FIG. 29). The secondary structure of ApTOLL consists of a main loop, herein named the "connector", different loops, and stems that connects the loops to the connector. The mutations can be located at the stems (A), connector (B) or loops (C). A substitution in a stem implies mutations in four nucleotides. If mutations are made in two different stems, 8 nucleotides are substituted consequently.

Furthermore, the effect of lengthening the 5' end and the 3' end of ApTOLL (SEQ ID NO: 7) and simultaneously introducing mutations was also tested. Three variants of SEQ ID NO: 7 were tested (see Table 16, below):

Aptamer 4F (SEQ ID NO: 19) (see FIG. 30): it has 13 extra nucleotides at the 5' end and 4 extra nucleotides at the 3' end compared to ApTOLL sequence (SEQ ID NO: 7). Sequence identity to SEQ ID NO: 7 would be 100% if only the central region was considered. Considering the entire sequence the percentage of sequence identity is 77.6%.

Aptamers 4F-Mut2 and 4F-Mut3 (SEQ ID NO: 20 and 21): these two aptamers would correspond to the most divergent sequences with respect to the ApTOLL sequence (SEQ ID NO: 7).

To test whether any 5' end extension or 3' end extension would work, the additional 5' end and 3' end regions of 4F-Mut2 and 4F-Mut3 extremely diverge from the extensions in aptamer 4F. The 5' extensions in 4F-Mut2 and 4F-Mut3 have **0% sequence identity** with respect to the 5' end extension of aptamer 4F. Likewise, the 3' extensions in 4F-Mut2 and 4F-Mut3 have **0% sequence identity** with respect to the 3' end extension of aptamer 4F.

	5' Additional Sequence	3' Additional Sequence
4F	GCGGATGAAGACT	CAAC
4F-Mut2	TTTTTCTTTTTTC	TTTT
4F-Mut3	ATAAGCAGGAGTC	TGGT

Table 16. The regions of 4F, 4F-Mut2 and 4F-Mut3 homologous to ApTOLL (SEQ ID NO: 7) are shaded in gray, while the mutations in 4F-Mut2 and 4F-Mut3 with respect to ApTOLL (SEQ ID NO: 7) are indicated by boldface.

Aptamer	5'-3' sequence	Sequence identity (%) Partial (Full)	Extra 5'	Extra 3'
ApTOLL (SEQ ID NO: 7)	GGTGTGCCAATAAACCATATCGCCGCG TTAGCATGTACTCGGTTGGCCCTAAAT ACGAG			
4F (SEQ ID NO: 19)	GCGGATGAAGACTGGTGTGCCAATAAA CCATATCGCCGCGTTAGCATGTACTCG GTTGGCCCTAAATACGAGCAAC	100% (77%)	13	4
4F-Mut2 (SEQ ID NO: 20)	TTTTTCTTTTTTCGGTGTGCCAATAAA CCAT GCT ACCGCGTTAGCATGTACTCG GTTGGCCTCAAATACGAGTTTT	90% (69.7%)	13	4
4F-Mut3 (SEQ ID NO: 21)	ATAAGCAGGAGTCGGTGTGCCAATAAACC ATGCT ACCGCGTTAGCATGTACTCGGT TGGCCTCAAATACGAGTGGT	90% (69.7%)	13	4

The ability of the above aptamers to inhibit TLR4 and the ability of the above aptamers to bind to TLR4 (antagonist activity against TLR4) were determined by means of assays disclosed above, and results are shown in Table 17.

5 Table 17. TLR4 antagonist activity of 4F, 4F-Mut2 and 4F-Mut3 aptamers.

Sample	Concentration	TLR4 activity (%)	Error (%)	TLR4 antagonist activity (%)
4F-Mut2	20 nM	74.4	3.0	Yes
	200 nM	69.8	5.0	
4F-Mut3	20 nM	77.6	4.0	Yes
	200 nM	79.0	8.0	
4F-T (Control)	20 nM	71.8	6.0	Yes
	200 nM	57.3	3.0	

Therefore, the addition of nucleotides at the 5' end and 3' end of SEQ ID NO: 7 does not affect formation of the effective binding structure and its activity, as confirmed experimentally in Table 17. The results from Table 17 confirm that both the 4F-Mut2 and the 4F-Mut3 aptamers, **which have**
10 **90% sequence identity with SEQ ID NO: 7 and also have the addition of 13 nucleotides at the 5' end and of 4 nucleotides at 3' end the aptamer of SEQ ID NO: 7**, maintain the capability of inhibiting TLR4 and the capability of binding to TLR4 (antagonist activity against TLR4), similar to their original aptamer of SEQ ID NO: 7.

15 Hence, a variant aptamer having at least 90% sequence identity with the original nucleic acid sequence (SEQ ID NO: 7) (or at least 69% sequence identity considering the whole sequence) and optionally extended at the 5' and 3' ends by 1-13 or 1-4 nucleotides, respectively, maintains the functions (i.e., the capability of binding specifically to and inhibiting TLR4) of the original nucleic acid sequence.

20 **LONG-TERM STABILITY OF APTAMERS**

EXAMPLE 11: Long-term stability study of ApSION 2 and ApSION 4

25 The objective of the study was to review that in general the performance values of aptamers that were obtained initially are maintained, this means, no sharp drop in purity, no significant change in the impurity profile, no sharp rises in impurities, and maintainance of the trend values of biological activity.

30 **11.1 Specifications**

- A Purity (% UV-area), impurity profile (peak % $\geq 0.1\%$ UV-area), total impurities (%), impurity with the highest % by IEX-HPLC method.

- Biological activity (% TLR4 activity) using the biological activity method.
- Where:
- Purity is % antagonist activity against TLR4.
 - % UV-area is the area under the curve of a given peak with respect to the total area identified by UV (sum of the areas of the identified peaks).
 - Impurity profile are the Impurities identified in the sample indicating the RRT and % UV-area.
 - Total impurities is 100% - % UV-main peak area.
 - Impurity with the highest % by IEX-HPLC method is the highest % UV-area identified in a peak that is not part of the main peak. RRT and % UV-area are indicated.
 - Biological activity expressed as % of antagonist activity against TLR4.
 - RRT: Relative retention time.
 - IEX-HPLC: Ion exchange High Performance Liquid Chromatography.

11.2 Methodology

- A total of 15 samples were prepared for the stability study. From which, six samples were analysed by “Ion Exchange High Performance Liquid Chromatography” (IEX-HPLC). Six more samples as replicates to be utilized in functional assays and three more replicates to be utilized as back- up. All samples were stored at the specified temperature of -20°C±5°C, stored in horizontal position.
- Table 18. Methodology of long-term stability study.

Test Method	Storage Conditions	Months & Dates					
Purity and Impurities by IEX-HPLC	-20°C ± 5°C	0	2	3	6	9	12

- The analysis of ApSION samples, for the determination of molecule stability, is a specific IEX-HPLC method developed internally by Sylentis SAU. The method consists of equilibrating the analysis column with two mobile phases in a gradient from 75% to 0% and re-equilibration to 75%, followed by injection of the respective analysis controls in the following sequence: blank, saline solution dilution, standard in saline, dilution saline, sample in saline diluted to the limit of detection; and finally the sample to be analyzed at the appropriate concentration for an accurate analysis.

11.3 Results and conclusion

- FIG. 31 show the stability parameters after twelve and nine months for ApSION 2 (A) and ApSION 4 (B), respectively. It can be said that ApSION 2 has proven to be stable for 12 months and ApSION 4 for 9 months in the storage condition of -20°C±5°C.

EXPERIMENTS WITH EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS (EAE) MOUSE MODEL

EXAMPLE 12: Efficacy study of ApTOLL, ApSION 2 and ApSION 4 in a preclinical model of multiple sclerosis

The aim of the study was to determine the effect of ApSION 2 and ApSION 4 in the clinical course of EAE and to define their optimal dose.

5 12.1 Materials and methods

 12.1.1 Guidelines and specification/justification

 The present study has been carried out according to the standard operating procedure in place at the test facility. All procedures were performed in compliance with the ARRIVE Guidelines, in accordance with the Guidelines of the European Union Council (63/2010/EU) following Spanish regulations (RD 53/2013, BOE 8/2(2013) for the use of laboratory animals. The generation of the EAE murine model of MS has been properly approved by institutional and regional ethics committees.

In vivo experiments cannot be replaced by *in vitro* systems because the ultimate goal is to study the demyelination and remyelination processes in an animal model of Multiple Sclerosis, as well as the effect in functionality measured as clinical score. The EAE model is the closest experimental approach to the biological and histopathological reality of this disease. There is no *in vitro* system to verify these neuropathological parameters.

 12.1.2 Materials

20 The main reagents used in this study are shown in Table 19.

Table 19. Specifications of the main reagents used.

Compound	Commercial House	Concentration	Use
MOG₃₅₋₅₅ (Myelin oligodendrocyte glycoprotein)	GenScript HK Limited (Hong Kong)	2 mg/ml	Immunization Day
Pertussis Toxin	Sigma	2,5 ng/ml	Immunization Day and 2 dpi
CFA: Incomplete Freund's adjuvant + Inactivated <i>M. tuberculosis</i> particles	Sigma H37RA Difco	4 mg/ml	Immunization Day
Ketamine (Anesketin)	Proyma	100 mg/ml	Immunization Day
Xylazine (Rompun)	Bayer	20 mg/ml	Immunization Day
Sodium pentobarbital (Dolethal)	Vetoquinol	50 mg/Kg	Euthanasia
OCT®	WVR Chemicals	-	Tissue freezing
Eriochrome-cyanine	Sigma	0,2%	Myelin staining
Borax	Sigma	0,01g/ml	Myelin staining

Potassium ferricyanide	Sigma	0,0125g/ml	Myelin staining
Proteome profiler mouse cytokine array kit	R&D Systems Inc.	-	Cytokines/chemokines expression

12.1.3 Protocols

12.1.3.1 EAE induction

The animals from the Charles River commercial house were acclimatized prior to carrying out the procedure for a week in the appropriate environmental conditions and in any case their feeding during the entire period of housing was *ad libitum*.

First, the necessary solutions for the induction of EAE under sterile conditions were prepared: the dissolution of MOG₃₅₋₅₅ at a concentration of 250 µg per mouse in 100 µl of sterile PBS1X; Freund's incomplete adjuvant was completed with inactivated *Mycobacterium tuberculosis* particles to reach a concentration of 4 mg/ml, obtaining Freund's complete adjuvant (CFA) and pertussis toxin, at a concentration of 400 ng per mouse in 100 µl of sterile PBS1X.

The animals (housed at 5 animals per cage) were anesthetized intraperitoneally with 40 µl of an anesthetic/analgesic mixture containing Ketamine and Xylazine, respectively. To avoid hypothermia, the animals were manipulated from this moment on a thermal blanket at 37 °C throughout the process.

To prepare the emulsion of CFA and MOG₃₅₋₅₅ a three-way wrench was used, connecting two 2 ml syringes with an equal volume of each reagent, according to the number of mice to be immunized, until obtaining a whitish emulsion of thick consistency. 200 µl of solution were injected subdermally into each animal divided equally (50 µl) into each groin and armpit.

After the MOG₃₅₋₅₅ injection, intravenous administration of the pertussis toxin was carried out in one of the lateral veins of the tail. To dilate the vein local heat was applied. The pertussis toxin was administered both on the day of immunization and 48 hours later. The animals used as controls for the validation of the model (sham group) were immunized in the same way except for MOG₃₅₋₅₅ peptide, which was replaced by sterile PBS.

The daily evaluation was carried out by two researchers visually, double blind and at the same time and place, following the criteria established by the laboratory based on the literature, giving the following affectation score: 0 = asymptomatic; 0.5 = paralysis of the distal tail; 1 = loss of muscle tone throughout the tail; 1.5 = flaccid tail and slight weakness in the hind legs; 2 = manifest weakness in the hind legs; 2.5 = total unilateral paralysis of a hind limb; 3 = bilateral paralysis of the hind limbs and weakness in the front; 3.5 = paraplegia and unilateral paralysis of the front limb; 4 = tetraplegia; 4.5 = dying animal and 5 = death. The mice were assessed every day in a double-blind way, from

the moment of immunization until sacrifice, collecting the weight data and evaluating the score they reached on the clinical scale.

12.1.3.2 Aptamers treatment

- 5 First, vials with the lyophilized aptamer were reconstituted in 100 μ l of sterile water. The vials were then shaken, centrifuged (so that the entire volume remained at the bottom of the tube) and left for 5 minutes at room temperature (RT) to stabilize.

- For the study of the immunomodulatory effect, ApSION 2 (0.23 mg/Kg, 0.45 mg/Kg, 0.91 mg/Kg and 1.82 mg/Kg), or ApSION 4 (0.45 mg/Kg, 0.91 mg/Kg and 1.82 mg/Kg), or ApTOLL (0.91 mg/Kg), or sterile PBS-1mM $MgCl_2$ (vehicle) was intravenously administered (in a volume proportional to the weight of each mouse) through the caudal vein in a single injection the first day they began to show symptoms. The vehicle is formed by a solution of $MgCl_2$ 1mM PBS (Phosphate-buffered saline). Animals were sacrificed 10 days later by intraperitoneal administration of a lethal dose of Dolethal® and were perfused transcardially.
- 10
15

12.1.3.3 Tissue processing

- The extraction and processing of nerve tissue was carried out 10 days post-onset (time of drug administration). Mice were administered with a euthanasic dose of Dolethal®, then transcardiac perfusion using 4% paraformaldehyde (PFA) was performed. Brain, cerebellum and spinal cord were obtained, washed three times for 10 min in PB (phosphate buffered), and cryoprotected by three successive passes in growing concentrations of sucrose in PB. Finally, tissue was frozen in OCT (WVR Chemicals), separated into different parts (brain, cerebellum, cervical spinal cord, thoracic spinal cord in two parts (T1 and T2) and lumbar spinal cord). T1 was used for this study and cut with a cryostat at a thickness of 20 micrometers in a transverse plane.
- 20
25

12.1.3.4 Eriochrome cyanine staining

- For the analysis of CNS demyelination, eriochrome-cyanine (EC) (Sigma), staining was performed. In this project, the histological sections were dried for 2 hours at room temperature (RT) and another 2 hours in a stove at 37°C. Then, the slides were immersed in cold acetone for 5 minutes at RT and allowed to aerate for 30 minutes for the acetone to evaporate. Subsequently, the cuts were immersed in a staining solution containing 0.2% Eriochrome-Cyanine, 0.5% sulfuric acid (H_2SO_4 , Sigma) and 4% ferric aluminum (Sigma, 10% prepared in distilled water), in distilled water for 30 minutes at RT. Excess dye was removed and the cuts were immersed in an aqueous solution of 5% ferric aluminum (Sigma) in distilled water for 10 minutes at RT for differentiation of dyed tissue. The excess dye was removed again with water, and slices were immersed in a solution of borax-ferrocyanide, for 10 minutes at RT. After washing with water, the staining can be observed under the light field microscope (myelinated areas stained in blue and white or yellowish demyelinated areas). For its conservation, the sections were dehydrated in ethanol solutions of increasing concentration at 70, 80, 90, 96 and 100%, rinsed in two 100% xylene baths and mounted with medium mounting.
- 30
35
40

12.1.3.5 Immunohistochemistry

For the detection of antigens present in the sections, they were allowed to defrost and dry at RT for 1 hour. Then, a pretreatment with 10% methanol in 0.1 M PB was performed at RT for 15 minutes and under stirring. After two washes of 0.1 M PB and 1X PBS of 10 minutes each, incubation was carried out in blocking solution to avoid nonspecific binding (5% of normal donkey serum (NDS; Millipore) and 0.02 % of Triton X-100 (Sigma-Aldrich) in 1X PBS, for one hour, in the dark, wet chamber and RT. After this, the sections were incubated in the mixture of primary antibodies (MBP (rat, Serotec), NFH (rabbit, Abcam), Olig2 (rabbit, Millipore) and PDGFR α (goat, Rd)) in the corresponding blocking solution for 12 hours, at 4 °C and in a humid chamber, development was performed by using secondary fluorescence antibodies in the blocking solution at a concentration of 1:1000 for one hour at RT. From this point on, the sections were stained with Hoechst (Sigma) for core development at a concentration of 1:10 relative to the stock (100 μ g/ml of Bisbenzimidazole, Sigma-Aldrich, in milliQ water) in PBS and for 10 minutes at RT, in darkness and in wet chamber. Finally, the sections were washed in PBS and mounted with cub Slides in Fluoromont® mounting medium (Southern Biotech).

12.1.3.6 Image and analysis

The inverted Leica SP-5 confocal microscope of the Microscopy and Image Analysis service of the Cajal Institute was used to take images of the spinal cord cuts. Three photos (mosaics) were taken per animal with a separation between planes of 3 μ m at a magnification of 40x and a resolution of 512 x 512 pixels. For the analysis of the area of NFH and MBP as well as the demyelinated area of EC staining, the Image J application was used. IMARIS Software for 3D and 4D Imaging was used to count oligodendroglial cells.

12.1.3.7 Cytokine array

The relative expression of 40 different cytokines/chemokines in plasma was determined using a proteome profiler mouse cytokine array kit (R&D Systems Inc., Minneapolis, MN, USA) and followed the provider's protocol. In brief, the membranes were first incubated for 1 hour on a horizontal platform shaker in blocking buffer. Samples were prepared by adding the plasma to 0.5 mL of array buffer 4 and adding 15 μ L of the Mouse Cytokine Array Panel A Detection Antibody Cocktail to the samples. The incubation was kept for 1h at RT. After this blocking step, blocking buffer was removed, the sample mix was added onto the membranes and incubated overnight at 4°C on a horizontal platform shaker. Finally, membranes were washed twice and then added 2 mL of a Streptavidin-HRP solution onto the membranes and incubated them for 30 min. Then, the membranes were washed again as before, and were put between a sheet where we added 1 mL of Chemi Reagent Mix. All air bubbles were smoothed out and incubation was performed for 1 min. Excess buffer was removed, the cover was reapplied and finally proteins were revealed by chemiluminescence.

12.1.4 Determinations

Following the regulations and ethics on animal experimentation, the criterion of 3Rs ('Replacement, Reduction and Refinement') was applied throughout the development of this study. In addition, the following endpoint criteria were established: any animal that reached a clinical evaluation score equal to or greater than 4 would be slaughtered; or showing signs of pain or stress for more than 48 hours even if the score was less than 3, taken as such verbalizations, presentation of stereotypes, lordo-
kyphosis, hair loss or weight loss greater than 2 g/day.

12.1.5 Statistical analysis

GraphPad Prism 9 software was used for statistical analysis. Data are expressed as the statistical mean $\pm$ the standard error of the mean. To compare pairs of independent groups, Student's *t*-test was used. For multiple comparisons, One-Way ANOVA test was carried out in conjunction with the corresponding *post hoc* test (if ANOVA was significant). The minimum level of statistical significance was $p < 0.05$, establishing the following degrees of significance: * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$.

12.2 Results and discussion

In this study, a single injection was made with different doses of ApSION 2 (0.23 mg/Kg, 0.45 mg/Kg, 0.91 mg/Kg, or 1.82 mg/Kg), or ApSION 4 (0.45 mg/Kg, 0.91 mg/Kg or 1.82 mg/Kg) and compared each group with 0.91 mg/Kg ApTOLL and the one that had been treated with the vehicle. The treatment was administered at the first time the animals showed clinical symptoms (onset).

Concerning symptomatology, under these experimental conditions, ApTOLL was the group that showed the best results, followed by ApSION 2 at 1.82 mg/Kg, and the effect was decreased as the dose was reduced (FIG. 33(A)). Additionally, the results of the dose-response study with ApSION 4 showed a greater effect with the intermediate dose used (0.91 mg/kg), where the curve was slightly lower than the other two doses tested (0.45 mg/kg and 1.82 mg/kg). Even so, the clinical curve with ApTOLL was below that the one obtained with ApSION 4 0.91 mg/kg (FIG. 33(B)). These results suggest a significant effect of both ApSION 2 and ApSION 4 reducing EAE damage.

A histological study with ApSION 2 at 0.45 mg/kg and ApSION 2 at 0.91 mg/Kg was performed, as it is the optimal dose of ApTOLL. First, an eriochrome-cyanine staining of thoracic spinal cord sections was performed to analyze the number and size of demyelinating lesions. It was observed that treatment with ApSION 2 (at both doses) decreased the percentage of demyelinated area within the white matter compared to their respective EAE-Veh group (FIG. 34).

In addition, differences were analyzed by studying a variety of myelin-related markers between the different groups. ApSION 2 treatment showed a significant improvement for the dose 0.45 mg/Kg in the area occupied by MBP, one of the main myelin proteins, with respect to vehicle group, approaching the percentage observed in the sham group. However, when compared to ApTOLL, the latter again produced a greater effect (FIG. 35). Regarding the study of neurofilaments (NFH marker), it

was observed that ApSION 2 is not only recovered against EAE-Veh (with the dose 0.45 mg/Kg, that reaches the values of sham group), but also produces the same improvement as ApTOLL (FIG. 36).

On the other hand, considering the differences produced in myelin area, it was evaluated whether this could be related to changes in the number of oligodendrocytes. For this purpose, the number of cells of the oligodendroglial lineage (Olig2-positive cells) was quantified. The ApSION 2 treatment showed a higher percentage of this cell type than the vehicle-treated animals, although it did not reach the values of the sham group. As for the comparison of the treatment with ApTOLL, no differences were found (FIG. 37). More specifically, changes in oligodendrocyte precursors cells (PDGFR α -positive cells) were analyzed, which are necessary in the repair of lesions for the generation of new myelin. Similar results to the previous one were observed, where ApSION 2 showed a higher percentage of cells compared to the vehicle, this time reaching the values of the control (FIG. 38).

In order to determine if there were also changes in inflammatory profile a cytokine/chemokines array was carried out. A first analysis was performed using plasma collected from mice treated with ApTOLL and compared with EAE-Veh animals, after 10 days post-onset, in order to analyze differences during symptom recovery. FIG. 39(A) shows the integrated density values of each sample of the set of molecules studied in this array. However, no significant differences were observed between groups in any of them. Despite these results, a cytokine study was performed after treatment of EAE animals with ApSION 2 at 0.45 mg/kg and compared them with their respective vehicles (FIG. 39(B)). In this case, significant differences were observed in a few molecules (BLC, G-CSF, ICAM-1, M-CSF, RANTES), in which the ApSION 2 treatment decreased their expression (FIG. 39(C)).

In conclusion, both ApSION2 and ApSION4 protect animals from disability in the EAE model by reducing the clinical score curve compared to vehicle-treated animals. In addition, histological analysis also showed an improvement in the preservation of myelin and neurofilaments, as well as in the percentage of oligodendroglial cells and oligodendrocyte.

REFERENCES

- WO2015/197706A1
WO2020/230108A1
5 WO2020/230109A1
U.S. Pat. No. 10,196,642
U.S. Pat. No. 8,404,649
U.S. Pat. No. 8,580,756
U.S. Pat. No. 8,163,708
10 U.S. Pat. No. 9,034,837
Basso DM, Beattie MS, Bresnahan JC. A sensitive and reliable locomotor rating scale for open field testing in rats. J Neurotrauma. 1995 Feb; 12(1):1-21.

CLAIMS

1. An aptamer comprising:
(a) a polynucleotide sequence consisting of:
5 i) SEQ ID NO: 7 or a fragment thereof consisting of SEQ ID NO: 8; or
 ii) a functionally equivalent variant having at least 70% sequence identity to SEQ ID NO: 7 or SEQ
 ID NO: 8, wherein the functionally equivalent variant:
 - has a length between 40 and 65 nucleotides;
 - has the capability of specifically binding to TLR-4 and inhibiting TLR-4 activation; and
10 - has the secondary structure of SEQ ID NO: 7 or of the fragment thereof, as determined using
 mFold;
(b) at least 10 chemically modified nucleotides which are sugar modified nucleotides; and
(c) optionally, the addition of at least one inverted nucleotide at the 3' and/or 5' end of the sequence;
wherein the aptamer has the capability of specifically binding to TLR-4 and inhibiting TLR-4 activa-
15 tion.
2. The aptamer according to claim 1, wherein each sugar modified nucleotide is independently se-
lected from the group consisting of: 2'-fluoro-2'-deoxynucleoside, 2'-O-methoxyethyl-nucleoside, 2'-
O-methylnucleoside, and 2'-O, 4'-C-methylene-β-D-ribofuranosyl nucleoside.
20
3. The aptamer according to any of claims 1-2, wherein the aptamer comprises 16, 23, or 29 chem-
ically modified nucleotides.
4. The aptamer according to any of claims 1-3, wherein the chemically modified nucleotide is a py-
25 rimidine.
5. The aptamer according to any of claims 1-4, wherein the aptamer comprises: i) at least 9 chemi-
cally modified T nucleotides, and ii) at least 7 chemically modified C nucleotides.
- 30 6. The aptamer according to any of claims 1-5, wherein the aptamer comprises between 1 and 5
additional nucleotides at the 3' end, wherein the additional nucleotide is an inverted nucleoside, par-
ticularly thymidinyl (3'-3') phosphate thymidine, or 2'-deoxycytidinyl (3'-3') phosphate 2'-deoxycyti-
dine.
- 35 7. The aptamer according to any of claims 1-6, wherein the chemically modified nucleotide is located
at any of residues 3, 5, 7, 8, 11, 15, 16, 18, 20, 21, 23, 24, 26, 28, 29, 32, 34, 36, 38, 39, 40, 43, 44,
47, 48, 49, 50, 54 and 56 of SEQ ID NO: 7.
- 40 8. The aptamer according to claim 7, wherein the chemically modified nucleotide is located at resi-
dues 7, 8, 11, 18, 20, 23, 24, 28, 29, 34, 38, 39, 40, 43, 44, and 47 of SEQ ID NO: 7.

9. The aptamer according to any of claims 1-8, wherein the aptamer comprises the same type of chemical modification throughout the entire aptamer.

5 10. The aptamer according to any of claims 1-9, wherein the aptamer is selected from the group consisting of SEQ ID NO: 1-6.

11. The aptamer according to any of claims 1-9, wherein the functionally equivalent variant has at least 90% sequence identity to SEQ ID NO: 7 and a length between 41 and 61 nucleotides.

10

12. An aptamer as defined in any of claims 1-11, for use as a medicament.

13. An aptamer as defined in any of claims 1-11, for use in the treatment of cancer, particularly breast cancer.

15

14. The aptamer for use according to claim 13, wherein the administration of the aptamer results in at least one outcome selected from the group consisting of:

- reduction in tumor progression;

- delayed disease progression;

20 - tumor shrinkage;

- tumor size reduction;

- reduction or prevention of tumor growth;

- inhibition or reduction of angiogenesis;

- inhibition or reduction of tumor invasion;

25 - inhibition or reduction of metastasis;

- improved survival rate;

- reduction in side effects;

- increase in quality of life;

- improved prognosis;

30 - enhanced response to therapy;

- improved disease-free survival;

- decrease in TLR-4 protein levels; and

- decrease in mRNA TLR-4 levels.

35 15. An aptamer as defined in any of claims 1-11, for use in the treatment of an inflammatory autoimmune neuropathy disorder, particularly Guillain-Barré Syndrome.

16. The aptamer for use according to claim 15, wherein the administration of the aptamer of the invention results in at least one outcome selected from the group consisting of:

40 - increase of neuromotor performance;

- increase of neuromuscular performance;
- increase of electrophysiological performance;
- increase of nerve conduction amplitude and velocity;
- decrease of plasma inflammatory cytokines;
- 5 - decrease of TLR-4 protein levels; and
- decrease of mRNA TLR-4 levels.