

“SITE-SPECIFIC CHIMERIC APTAMERS AND USES THEREOF”

FIELD OF THE INVENTION

The present invention concerns the field of chimeric aptamers with high gene
5 target specificity suitable for RNA based therapy.

In particular, the present invention relates to the use of the chimeric aptamers
for the preparation of a pharmaceutical composition for treating diseases
characterized by altered DNA methylation.

STATE OF THE ART

- 10 DNA methylation is a key epigenetic modification that mainly occurs within CpG
dinucleotides and is mediated by the members of the DNA methyltransferase
(DNMT) family (DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L).
Numerous studies have established a link between aberrant promoter DNA
methylation and cancer. Aberrant epigenetic modifications which occur in
15 pathological situations are reversible offering a unique possibility to alter gene
function reprogramming and malignant cellular transformation. In this view,
epigenetic targeting by specific and effective inhibitors could lead to the
development of clinically relevant strategies for the therapy of cancer and other
pathologies characterized by altered methylation.
- 20 Aptamers are small (6-30kD) synthetic nucleic acids functioning as high affinity
ligands. They are selected through a process known as SELEX (systematic
evolution of ligands by exponential enrichment) that relies on the ability of the
target protein to select high-affinity ligands from a random pool of nucleic acids.
Besides being cost-effective and relatively easy to manipulate, aptamers
25 demonstrate high affinity for their targets (in the low nanomolar range) and
specificity similar to monoclonal antibodies and high tissue penetration
comparable to small molecules. Moreover, aptamers are neither immunogenic
nor toxic. All these features make them ideal candidate molecules for both
diagnostic and clinical applications.
- 30 WO 2021/086266 describes RNA-based demethylating molecules, named

aptaDiRs, which combine the low cytotoxicity proper to modified RNAs. Since aptaDiRs have the ability to selectively bind and inhibit the DNMT1 enzyme, they have a very broad demethylation action and result in a broad and non-specific gene activation in cancer cells, since aptaDiRs act on the ubiquitous
5 DNMT1 enzyme.

Due to the non-specific action of currently available solutions, there is a need to provide alternative aptamer-based drugs for RNA based therapy, having a higher targeting specificity compared to those already available, for use in the preparation of new drugs for treating cancer and other pathologies characterized
10 by altered methylation.

SUMMARY OF THE INVENTION

The problem underlying the present invention is that of making available site-specific chimeric aptamer: captaDiRs (chimeric aptaDiRs) capable of reverting site-specific hypermethylation that cause gene silencing, for the manufacture of
15 medicaments destined for the therapy of cancers as well as neurological, neurodegenerative and rare diseases.

This problem is resolved by the present finding by the use of site-specific chimeric aptamer capable of inhibiting DNA methyltransferases in specific genomic locations. Unlike aptaDiRs, that are unable to target a specific DNA
20 region, captaDiRs are designed to selectively bind the regulatory regions of target genes by forming a triple-helix or R-loop.

The present invention thus concerns a site-specific chimeric aptamer (captaDiR) capable of inhibiting DNA methyltransferase 1 (DNMT1) at a specific target gene locus, wherein the chimeric aptamer comprises:

- 25 - a first domain that is an oligonucleotide (DNA, RNA or modified RNA) with a sequence deriving from aptaDiR or aptaDiR like sequences that forms a stem loop structure RNA that is capable of interacting with DNMT1; and
- a second domain that is either

a gene-specific oligonucleotide (DNA, RNA or modified RNA) with a sequence able to anchor the aptamer to the specific target gene locus DNA sequence by forming an R loop with the DNA sequence of the target gene locus,
or

- 5 a gene-specific oligonucleotide (DNA; RNA or modified RNA) with a sequence able to anchor the aptamer to the specific target gene locus DNA sequence by forming a triplex forming sequence DNA sequence of the specific target gene locus;

wherein said second domain is fused to the 5', or to the 3', of the stem loop structure RNA; and wherein said site-specific chimeric aptamer protects said
10 target gene locus from DNMT1-mediated methylation, allowing the local activation of gene expression.

The invention further relates to a pharmaceutical composition comprising one or more site-specific chimeric aptamers according to the present disclosure and
15 one or more pharmaceutically acceptable excipients.

In a further aspect the invention relates to a site-specific chimeric aptamer capable of inhibiting DNA methyltransferase 1 (DNMT1) at a specific target gene locus according to the present disclosure or a pharmaceutical composition comprising them, for use as a medicament.

- 20 In a still further embodiment, the invention describes a site-specific chimeric aptamer capable of inhibiting DNA methyltransferase 1 (DNMT1) at a specific target gene locus according to the present disclosure or a pharmaceutical composition comprising them, for use in the treatment of cancer, neurological, neurodegenerative, and rare diseases including Fragile X syndrome (FXS),
25 Alzheimer's disease and Alzheimer -like disorders and rare diseases, including Fragile X syndrome (FXS), characterized by aberrant or altered DNA methylation.

BRIEF DESCRIPTION OF THE DRAWINGS

- The characteristics and advantages of the present invention will be apparent
30 from the detailed description reported below, from the Examples given for

illustrative and non-limiting purposes, and from the annexed Figures 1-7.

Figure 1: Model of action of captaDiR. The demethylating aptaDiR (Red) is fused to a sequence (Blue) corresponding to the targeted gene locus. The DNMT1 is inhibited only locally restoring the expression of the gene of interest.

- 5 Figure 2: Triplex structure. a) scheme of Triplex and R-loop structure. b) RNA can assume triple RNA-DNA helix formation. Upper panel: Diagram showing position of the DNA and RNA oligonucleotides. Bottom right panel: examples of RNA:DNA hybrids, reverse Hoogsteen (RH triplex) and R loop. Bottom left panel: Results of annealing double-stranded (ds) DNA (probe) with single-stranded (ss) DNA and RNAs; 32P-labeled ds DNA probe (DT12) was annealed with the respective ss oligonucleotide and the mixture was separated on PAGE.
- 10 Figure 3: Triplex helix forming oligonucleotides ability to anchor *CEBPA*. Upper panel: scheme of the construct. The sequences were engineered under the T7 polymerase promoter in order to anchor CEBPA and guide T7 polymerase local activity. Lower panel: In the presence of T7-RNA polymerase, CEBPA transcript was increased.
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- Figure 4: CEBPA targeting captaDiR. a) CaptaDiRs. Ce-49 sh aptaDiR (right) is fused to a CEBPA targeting sequence (left). XXXX indicates a covalent spacer, a 4 carbon linker; b) CEBPA and GAD1 levels by RT-qPCR in K562 cells transfected for 72 hours with 100 nM a control (Cont.) or CEBPA-targeted Ce-49 sh (Ce-49-CEBPA).
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- Figure 5: Gene-specific conjugate. a) CEBPA-targeted captaDiR is linked to a carrier aptamer by the use of sticky complementary sequences. b) A549 cells (Axl positive) were left untreated (NT) or treated with 500nM anti-Axl carrier aptamer (GL21.T) or GL21.T linked to Ce-49-CEBPA captaDiR (Chimera). Levels of CEBPA were analysed by RT-qPCR.
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Figure 6: Cell viability upon captaDiR transfection. A549 cells were left untreated (NT) or transfected with 100 nM control sequence (Ctrl), Ce-49 sh and CEBPA captaDiR. Cell viability was measured after 72 hours.

Figure 7: FMR1 reactivation after aptaDiR transfection of FXS fibroblasts. Reactivation of FMR1 one week after treatment with aptaDiR added either twice or three times, left panel; no reactivation observed using Azacitidine, right panel. AptaDiR treatment: 100 nM transfection.

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DETAILED DESCRIPTION OF THE INVENTION

Known RNA-based demethylating molecules named aptaDiRs, combine two features: low cytotoxicity of modified RNAs, and the ability to selectively bind and inhibit the DNMT1 enzyme (WO2021086266) causing a broad demethylation and gene activation in cancer cells.

The present invention relates to an innovative approach to generate chimeric RNAs (site-specific chimeric aptamers), named captaDiRs (chimeric aptaDiRs). captaDiRs are improved aptaDiRs, which have been elongated and include a targeting sequence which is able to guide the aptamer to a specific gene locus (Figure 1).

The captaDiRs allow to: 1) anchor themselves to a specific genomic location, 2) inhibit DNMT1 enzymatic activity thereby protecting the locus from DNMT1-mediated methylation, allowing the local activation of gene expression.

CaptaDiRs represent a novel RNA-based-platform to control DNA methylation in predefined loci and have surprisingly been found efficient in the treatment of diseases such as cancers and any disease associated to methylation-dependent deficiency of key proteins.

Furthermore, as compared to the well-known aptamer-based chimeric drugs, captaDiRs hold a unique advantage as epigenetic regulators. Indeed, captaDiRs have a high target specificity for nuclear functions (Fig 4) due to the fact that the aptaDiR moiety has the capability of entering the nucleus fused to the anchoring moiety, thus carrying out the aptamer-dependent enzyme inhibitory function only locally upon anchoring the construct to a genomic site.

As such, the captaDiRs according to the present invention permit a shift toward a safe and effective targeted therapy for a wide range of different diseases.

The present invention therefore concerns a site-specific chimeric aptamer (captaDiR) capable of inhibiting DNA methyltransferase 1 (DNMT1) at a specific target gene locus, wherein the aptamer comprises:

- a first domain that is an oligonucleotide (DNA, RNA or modified RNA) with a sequence deriving from an aptaDiR or aptaDiR like sequences that by assuming a stem loop structure RNA is capable of interacting with DNMT1; and
 - a second domain that is either
 - a gene-specific oligonucleotide (DNA, RNA or modified RNA) with a sequence able to anchor the aptamer to the specific target gene locus DNA sequence by forming an R loop with the DNA sequence of the desired target gene locus,
 - or
 - a gene-specific oligonucleotide (DNA, RNA or modified RNA) with a sequence able to anchor the aptamer to the specific target gene locus DNA sequence by forming a triplex forming sequence with the DNA sequence of the desired target gene locus;
- wherein said second domain is fused to the 5', or to the 3', of the stem loop structure RNA; and wherein said site-specific chimeric aptamer protects said target gene locus from DNMT1-mediated methylation, allowing the local activation of gene expression.

- Between the first and second domains of the captaDiR there may be a covalent spacer which is preferably a 4 carbon linker.

Definitions (as used herein):

- the term "aptamer" refers to a short oligonucleotide RNA that binds a specific target molecule;
- the term "aptaDiR" refers to aptamers that bind to and inhibit the DNMT1 enzyme.

"aptaDiR" has the sequence that is at least 70% sequence identity to sequences selected from the group consisting of

SEQ ID NO:31 5' CUGAGCUCAUGGCGAGGCUUCU 3',

- SEQ ID NO:32 5' UGGGCUGAGCUCAUGGCGAGGCUUC 3',

- SEQ ID NO: 33 5' CUGAGGCCUAACGAAGGCUUCU 3',
 SEQ ID NO:34 5' CUGAGGUAAUGGCGAGGCUUCU 3',
 SEQ ID NO:35 5' AGGUAAUGGCGAGGCUUCUUAUCUG 3',
 SEQ ID NO:36 5' UUACUGGGCUGAGGUAAUGGCGAGG 3', and
 5 SEQ ID NO:37 5' CTGAGGTAATGGCGAGGCTTCT 3'.

Sequences of SEQ ID Nos 31-37 are RNA sequences. Uracil "U" may be replaced by thymine "T" or *viceversa*.

- 10 Preferably the aptaDiR (first domain) has a sequence as set forth in SEQ ID NO:33: 5' CUGAGGCCUAACGAAGGCUUCU 3';

- the term "stem loop structure" refers to a base pairing pattern that can occur in single-stranded RNA, also known as a hairpin or hairpin loop and occurs when two regions of the same strand, usually complementary in nucleotide sequence when read in opposite directions, base-pair to form a double helix that ends in an unpaired loop;

- the term "triplex" refers to a three-stranded nucleic acid structure, composed of a DNA:RNA or DNA: DNA duplex and the associated non-template single-stranded DNA (see Figure 2a);

- the term R-loop refers to a three-stranded nucleic acid structure composed of an RNA:DNA hybrid and a displaced single-stranded DNA (see Figure 2a); and

- the term "Hoogsteen base pairing" refers to a variation of base-pairing in nucleic acids such as the A-T pair. In this manner, two nucleobases, one on each strand, can be held together by hydrogen bonds in the major groove. In some cases, called "reversed Hoogsteen base pairing", one base is rotated 180° with respect to the other (see Figure 2).

In a preferred aspect, the site-specific chimeric aptamer according to the invention is capable of reducing DNMT1 function by at least about 10%, preferably by at least about 20%, more preferably by at least about 30%, and/or the aptamer is capable of increasing the target gene expression level.

In the site-specific chimeric aptamer of the present invention the following preferred aspects can be present:

- the first domain of the chimeric aptamer comprises a stem-loop structure comprising two or more pairs of nucleotides;
- 5 - the first domain of the chimeric aptamer preferably has a sequence as set forth in SEQ ID NO:33;
- second domain is formed by four or more nucleotides;
- the site-specific chimeric aptamer has a length in the range from 15 to 55 nucleotides, preferably in the range from 30 to 55 nucleotides, more preferably
- 10 it has a length of 52 nucleotides;
- the site-specific chimeric aptamer has a covalent spacer, preferably a 4 carbon linker, between the first and second domains;
- said site-specific chimeric aptamer has any chemical and/or structural modifications, including a chimeric aptamer consisting of multiple copies of the
- 15 aptamer domain fused to one or more targeting domains.

The site-specific chimeric aptamer according to the present invention has a sequence that depends on the target gene, preferably the gene specific sequence (second domain) of the captaDiR has a length in the range from 16 to 35 nucleotides. When it comprises a triplex forming sequence, the captaDiR

20 second domain sequence is preferably either poly(Pu) or poly(Py) complementary to the target gene sequence.

In a preferred aspect the captaDiR is specific for a target gene chosen from the group consisting of: CEBPA (CCAAT enhancer binding protein alpha), SOCS3 (suppressor of cytokine signaling 3) and FMR1 (fragile X messenger

25 ribonucleoprotein 1).

In a preferred aspect the captaDiR has a target sequence of:

For the CEBPA target gene:

SEQ ID NO:1 TFO_RH_CEBPA 5'-GGCGAAGGAGGGGGCGGAGGGGTAAG-3'

SEQ ID NO:2 TFO_RH2_CEBPA 5'-CCTAGGGCAGGCAGGAGGAGGGG-3' (original

30 CEBPA sequence)

SEQ ID NO:3 RL_1CEBPA 5'-GAATGGGGAGGCGGGGGAGGAAGCGG-3'

For the SOCS3 target gene:

SEQ ID NO:4 TFO_H_1SOCS3

5'-GGCGGGGAGGGGGTAGAGGGGAGGAGAGGAAGTGA-3'

5 SEQ ID NO:5

TFO_RH_1SOCS3 5'-CCGCCCCTCCCCATCTCCCCTCCTCTCCTTCACT-3'

SEQ ID NO:6

RL_1SOCS3 5'-TCACTTCTCTCCTCCCCTCTACCCCTCCCCGCC-3'

SEQ ID NO:7 TFO_H_2SOCS3 5'-TCTTCCCTCCCCCACCCTTTTCACTT-3'

10 SEQ ID NO:8 TFO_RH_2SOCS3

5'-AAGTGTGAAAGGGGGGTGGGGGAGGGAAGA-3'

SEQ ID NO:9 RL_2SOCS3 5'-AGAAGGGAGGGGGGTGGGGGAAAGTGTGAA-3'

SEQ ID NO:10 TFO_H_3SOS3 5'-GAGCGCGGAGGGTGAGGGAGGGG-3'

SEQ ID NO:11 TFO_RH_3SOS3 5'-CTCGCGCCTCCCACTCCCTCCCC-3'

15 SEQ ID NO:12 RL_3SOCS3 5'-CCCCTCCCTCACCTCCGCGCTC-3'

For the FMR1 target gene:

SEQ ID NO:13 TFO_H_1FMR 5'-CCACTCTCTCCCGCATCT-3'

SEQ ID NO:14 TFO_RH_1FMR 5'-AGATGCGGGAGAGAGTGG-3'

SEQ ID NO:15 RL_1FMR 5'-GGTGAGAGAGGGCGTAGA-3'

20 SEQ ID NO:16 TFO_H_2FMR 5'-CTTCTCTCTCCCGCCCC-3'

SEQ ID NO:17 TFO_RH_2FMR 5'-GGGGCGGGAGAGAGAAG-3'

SEQ ID NO:18 RL_2FMR 5'-GAAGAGAGAGGGCGGGG-3'

SEQ ID NO:19 TFO_H_3FMR 5'-CGCCCCCTCCCTTGTCGC-3'

SEQ ID NO:20 TFO_RH_3FMR 5'-GCGACAAGGGAGGGGGGCG-3'

25 SEQ ID NO:21 RL_3FMR 5'-GCGGGGGGAGGGAACAGCG-3'

SEQ ID NO:22 TFO_H_4FMR 5'-CTTCTTTCTACCTCCTC-3'

SEQ ID NO:23 TFO_RH_4FMR 5'-GAGGAGGTAGAAGAGAAG-3'

SEQ ID NO:24 RL_4FMR 5'-GAAGAGAAGATGGAGGAG-3'

SEQ ID NO:25 TFO_H_5FMR 5'-CCACCTTCACGCCCC-3'

30 SEQ ID NO:26 TFO_RH_5FMR 5'-GGGGCGTGAAGGTGG-3'

SEQ ID NO:27 RL_5FMR 5'-GGTGGAAGTGCGGGG-3'

SEQ ID NO:28

TFO_H_6FMR 5'-TTCTTCTTTTCCCTCCCTTCCTTCCCGCTTCTACCCC-3'

SEQ ID NO:29

TFO_RH_6FMR5'-GGGGTAGAAGCGGGAAGGAAGGGAGGGAAAAGAAGAA-3'

SEQ ID NO:30

RL_6FMR 5'-CCCCATCTTCGCCCTTCCTTCCTCCCTTTTCTTCTT -3'

5 Other sequences are set forth as follows:

SEQ ID NO:38 AGGGCAGGCAGGAGGAGGGGG RNA sequence of ss RNA oligonucleotide RT12

SEQ ID NO:39 CGGGGAGGAGGAGAGGGAGUC RNA sequence of ss RNA oligonucleotide Rh12

10 SEQ ID NO:40 TCCCGTCCGTCTCCTCCCC Genomic DNA sequence of ds DNA oligonucleotide DT12

SEQ ID NO:41 GCCCCTCCTCCTCTCCCTC Genomic DNA sequence of ss DNA oligonucleotide corresponding to the upper strand

15 SEQ ID NO:42 CUGAGGGAGAGGAGGAGGGGC Reverse Hoogsteen pairing (RH triplex) between DT12 and Rh12

SEQ ID NO:43 GGGGAGGAGGAGAGGGAG DT3

SEQ ID NO: 44 catgTAATACGACTCACTATAGGGgaatggggaggcggggaggaagcgg Composite oligonucleotide T7 (CoT7-50)

20 SEQ ID NO: 45 gtacATTATGCTGAGTGATATCCC Composite oligonucleotide T7 (CoT7-50)

SEQ ID NO:46 corresponds to the CEBPA CaptaDiR, having the sequence:

GAATGGGGAGGCGGGGGAGGAAGCGGXXXXCUGAGGCCUAACGAAGGCUUCU

where XXXX is a spacer region which corresponds to a covalent spacer, a 4 carbon linker.

25 The invention further relates to a pharmaceutical composition comprising one or more site-specific chimeric aptamers according to the present disclosure and one or more pharmaceutically acceptable excipients.

So far, two FDA approved compounds (5-Azacytidine and 5-Aza 2' deoxycytidine) inhibit DNMTs and reduce global DNA methylation. However, they lack

30 of selectivity and show high toxicity and chemical instability (Doi: 10.1126/science.aaj2239). Therefore, there is a need to develop smart and safe epigenetic drugs able to modulate the expression of specific genes. The

CaptaDiRs of the present invention allow to solve the above lamented problem and enable reverting aberrant DNA methylation in a gene-specific modality and consequently gene-specific silencing. This specific modulation of gene expression has been surprisingly seen effective in the treatment of pathological conditions to which abnormal DNA methylation is associated, namely: cancer, neurological, neurodegenerative and rare genetic disorders.

In a further aspect the invention relates to a site-specific chimeric aptamer capable of inhibiting DNA methyltransferase 1 (DNMT1) at a specific target gene locus according to the present disclosure or a pharmaceutical composition comprising them, for use as a medicament.

In a still further embodiment, the invention describes a site-specific chimeric aptamer capable of inhibiting DNA methyltransferase 1 (DNMT1) locally at target gene locus according to the present disclosure or a pharmaceutical composition comprising them, for use in the treatment of cancer, neurological, neurodegenerative, and rare diseases characterized by aberrant or altered DNA methylation.

As used herein, conditions/diseases characterized by changes in DNA methylation or aberrant DNA methylation may include, but is not limited to, aging, including Alzheimer's disease and Alzheimer -like disorders , aberrant proliferative diseases such as cancer, autoimmune disease, genetic disorders, metabolic disorders, psychological disorders, imprinting disorders related to genetic syndrome as Beckwith-Wiedeman Syndrome (BWS), Prader Willi Syndrome (PWS), Angelman Syndrome (AS), Albright hereditary osteodystrophy (AHO), Pseudohypoparathyroidism type 1A (PHP-IA), Pseudohypoparathyroidism type 1B (PHP-IB); loss of imprinting (LOI) considered the most abundant and most precocious alteration in cancer i.e. LOI in IGF2/H19 in Wilms' tumor; repeat instability diseases wherein expansion of trinucleotide (TNR) repeats leads to silencing of the associated genes as observed in Fragile X Syndrome and Myotonic Dystrophy.

The potency and the gene specificity of the captaDiR will be test to correct aberrant gain of DNA methylation within a specific gene locus and/or to restore the expression of parental allele normally imprinted whereas the expressed allele is mutated, deleted for a particular locus or in case of Uniparental Disomy (UPD)

5 The term "cancer" encompasses a disease involving both pre-malignant and malignant cancer cells. In some examples, cancer refers to a localized overgrowth of cells that has not spread to other parts of a subject, i.e., a benign tumor. In other examples, cancer may be referring to a malignant tumor, which

10 has invaded and destroyed neighboring body structures and spread to distant sites. In some example, the disease characterized by aberrant DNA methylation may include, but is not limited to, acute lymphoblastic leukemia, acute myeloid leukemia, adrenocortical carcinoma, AIDS-related cancer, AIDS-related lymphoma, anal cancer, appendix cancer, astrocytomas (childhood cerebellar or cerebral), basal cell carcinoma, bile duct cancer, bladder cancer, bone

15 cancer, brainstem glioma, brain tumors (cerebellar astrocytoma, cerebral astrocytoma/malignant glioma, ependymoma, medulloblastoma, supratentorial primitive neuroectodermal tumors, visual pathway and hypothalamic gliomas), breast cancer, bronchial adenomas/carcinoids, Burkitt lymphoma, carcinoid

20 tumors (childhood, gastrointestinal), carcinoma of unknown primary, central nervous system lymphoma (primary), cerebellar astrocytoma, cerebral astrocytoma/malignant glioma, cervical cancer, childhood cancers, chronic lymphocytic leukemia, chronic myelogenous leukemia, chronic myeloproliferative disorders, colon cancer, cutaneous T- cell lymphoma,

25 desmoplastic small round cell tumor, endometrial cancer, ependymoma, esophageal cancer, Ewing's sarcoma in the Ewing family of tumors, extracranial germ cell tumor (childhood), extragonadal germ cell tumor, extrahepatic bile duct cancer, eye cancers (intraocular melanoma, retinoblastoma), gall bladder cancer, gastric (stomach) cancer, gastrointestinal carcinoid tumor,

30 gastrointestinal stromal tumor, germ cell tumors (childhood extracranial,

extragonadal, ovarian), gestational trophoblastic tumor, gliomas (adult, childhood brain stem, childhood cerebral astrocytoma, childhood visual pathway and hypothalamic), gastric carcinoid, hairy cell leukemia, head and neck cancer, hepatocellular (liver) cancer, Hodgkin lymphoma, hypopharyngeal cancer, 5 hypothalamic and visual pathway glioma (childhood), intraocular melanoma, islet cell carcinoma, Kaposi sarcoma, kidney cancer (renal cell cancer), laryngeal cancer, leukemias (acute lymphoblastic, acute myeloid, chronic lymphocytic, chronic myelogenous, hairy cell), lip and oral cavity cancer, liver cancer (primary), lung cancers (non-small cell, small cell), lymphomas (AIDS-related, Burkitt, cutaneous T -cell, Hodgkin, non-Hodgkin, primary central 10 nervous system), macroglobulinemia (Waldenstrom), malignant fibrous histiocytoma of bone/osteosarcoma, medulloblastoma (childhood), melanoma, intraocular melanoma, Merkel cell carcinoma, mesotheliomas (adult malignant, childhood), metastatic squamous neck cancer with occult primary, mouth 15 cancer, multiple endocrine neoplasia syndrome (childhood), multiple myeloma/plasma cell neoplasm, mycosis fungoides, myelodysplastic syndromes (MDS), myelodysplastic/myeloproliferative diseases, myelogenous leukemia (chronic / chronic myelogenous leukemia (CML), myeloid leukemias (adult acute, childhood acute), multiple myeloma, myeloproliferative disorders 20 (chronic), nasal cavity and paranasal sinus cancer, nasopharyngeal carcinoma, neuroblastoma, non-Hodgkin lymphoma, non-small cell lung cancer, oral cancer, oropharyngeal cancer, osteosarcoma/malignant fibrous histiocytoma of bone, ovarian cancer, ovarian epithelial cancer (surface epithelial-stromal tumor), ovarian germ cell tumor, ovarian low malignant potential tumor, 25 pancreatic cancer, pancreatic cancer (islet cell), paranasal sinus and nasal cavity cancer, parathyroid cancer, penile cancer, pharyngeal cancer, pheochromocytoma, pineal astrocytoma, pineal germinoma, pineoblastoma and supratentorial primitive neuroectodermal tumors (childhood), pituitary adenoma, plasma cell neoplasia, pleuropulmonary blastoma, primary central nervous 30 system lymphoma, prostate cancer, rectal cancer, renal cell carcinoma (kidney

cancer), renal pelvis and ureter transitional cell cancer, retinoblastoma, rhabdomyosarcoma (childhood), salivary gland cancer, sarcoma (Ewing family of tumors, Kaposi, soft tissue, uterine), Sezary syndrome, skin cancers (nonmelanoma, melanoma), skin carcinoma (Merkel cell), small cell lung cancer, small intestine cancer, soft tissue sarcoma, squamous cell carcinoma, squamous neck cancer with occult primary (metastatic), stomach cancer, supratentorial primitive neuroectodermal tumor (childhood), T Cell lymphoma (cutaneous), testicular cancer, throat cancer, thymoma (childhood), thymoma and thymic carcinoma, thyroid cancer, thyroid cancer (childhood), transitional cell cancer of the renal pelvis and ureter, trophoblastic tumor (gestational), unknown primary site (adult, childhood), ureter and renal pelvis transitional cell cancer, urethral cancer, uterine cancer (endometrial), uterine sarcoma, vaginal cancer, visual pathway and hypothalamic glioma (childhood), vulvar cancer, Waldenstrom macroglobulinemia, and Wilms tumor (childhood).

In some examples, the disease characterized by aberrant DNA methylation may be cancer. In some examples, the cancer may be chronic myelogenous leukemia (CML), Myelodysplastic syndrome (MDS) and/or non-small cell lung cancer (NSCLC) including adenocarcinoma (such as human alveolar basal epithelial adenocarcinoma), squamous cell carcinoma, and/or its combination thereof.

Cancer therapy cancers include those related to C/EBP alpha expression, such as myelodysplastic syndrome (e.g., acute myeloid leukemia or a myeloproliferative neoplasm), head and neck cancer, liver cancer (e.g., hepatoma and hepatocellular carcinoma), lung cancer (e.g., adenocarcinoma and non-small cell lung cancer), prostate cancer (e.g., adenocarcinoma), and skin cancer (e.g., squamous cell carcinoma).

Cancers where aberrant methylation of specific genes is related with the cancerous progression and/or advantageous phenotype such as: RB in retinoblastoma, CDKN2A (INK 4A and ARF transcript) in lung and colon cancer, CDH1 in breast, gastric thyroid leukemia, liver cancer, CDH13 in lung, ovarian,

pancreatic cancer, TIMP3 in brain and kidney cancer, VLH in renal carcinoma, MLH in colon, endometrial and gastric cancer, MGMT in brain, colon, lung and breast cancer, BRCA1 in breast and ovarian cancer, GSTP1 prostate, liver, colon, breast, and kidney cancer, SMARCA3 in colon and gastric cancer, 5 RASSF1 in lung, liver and brain cancer, SOCS1 in liver, colon and multiple myeloma, ESR1 in colon, breast estrogen receptor-negative, lung and leukemia, DAPK1 in lymphoma.

Advantageously the site-specific chimeric aptamers of the invention show many 10 advantages in the treatment of pathologies associated to the specific alteration of DNA methylation.

The CaptaDiR approach has a wide applicability to several pathologies including neurological, neurodegenerative and rare diseases associated to site-specific DNA methylation abnormalities, such as Fragile X Syndrome (FXS). FXS is due 15 to the expansion (> 200 units) of a CGG tract in exon 1 of the FMR1 gene (FM, full mutation) and consequent cytosines methylation (MFM, methylated FM), leading to transcriptional silencing of the gene and absence of the protein product.

The high binding capability of the chimeric aptamers renders them very 20 attractive molecules for clinical applications. Indeed, the chimeric aptamers show cost-effectiveness, high stability and may be easily modified for their *in vivo* use, providing highly selective, chemically stable and molecularly adaptable demethylating tools for reactivation of specific genes.

Despite the therapeutic potential of DNA methylation targeting, no new 25 hypomethylating therapies have been approved in the last decades. The results herein described provide the first example of an RNA-guided specific epigenetic therapy, holding a great promise for a personalized and less toxic targeted therapy.

The chimeric aptamers offer new effective tools with many important 30 advantages over the already existing hypomethylating-based protocols: 1) a

reduced toxicity; 2) absence of unwanted off-target effects; 3) high target gene specificity. The ability to control the expression of the individual genes will carry the promise of permitting a fundamental shift toward a personalized therapy.

Various embodiments and aspects of the present invention as delineated
5 hereinabove and as claimed in the claims section below find experimental support in the following examples for which commercial cell lines were used.

Furthermore, when the invention envisaged the use of primary cells derived from patients affected by FXS, the same were supplied by the Catholic University of the Sacred Heart and obtained in compliance with the informed consent of the
10 patients and with the approval of the relevant ethics commission.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions illustrate some embodiments of the invention.

Example 1.

15 The inventors of the present application have previously isolated a panel of DNMT1 -specific RNA aptamers- with high stability, able to inhibit DNMT1 activity (WO 2021/086266).

The present application relates to a novel and more efficient RNA-based-platform to control DNA methylation in predefined *loci*, the CaptaDiR approach.

20 As a proof of principle, the CEBPA gene (CCAAT Enhancer Binding Protein Alpha) was used. CEBPA encodes a transcription factor that contains a basic leucine zipper (bZIP) domain and recognizes the CCAAT motif in the promoters of target genes.

As shown in Figure 2b, using *CEBPA* as a proof-of-principle target, regions of
25 the extra-coding region of the gene (ecCEBPA) that can form triplex structures were identified.

In order to demonstrate the ability of such regions to anchor *CEBPA*, the sequences were engineered under the T7 polymerase promoter. It was found that in the presence of the T7 polymerase, *CEBPA* transcript was increased
30 indicating the ability of the identified regions to bind the gene locus guiding T7

polymerase local activity (Figure 3).

Most importantly, a chimeric RNA was designed by fusing the 5'-end of one aptaDiR (Ce-49 sh) to one identified CEBPA-targeting sequence (Ce-49-CEBPA) (Figure 4a). This construct retains the ability to upregulate CEBPA upon transfection (Figure 4b) as Ce-49 sh aptaDiR in K562 cells.

Most importantly, it does not upregulate GAD1 gene that is instead upregulated by Ce-49 sh (Figure 4b).

Furthermore, we demonstrated that it is possible to conjugate the Ce-49 sh-CEBPA captaDiR to a carrier aptamer by a sticky based strategy (Figure 5a).

As proof of concept, a nuclease-resistant RNA aptamer named GL21.T was used, which binds and inhibits Axl receptor overexpressed in several tumour types including non-small cell lung cancer (NSCLC). This aptamer was generated and characterized by our group and widely applied for the selective delivery of therapeutic RNAs.

It was demonstrated that GL21.T is able to efficiently deliver Ce-49-CEBPA to Axl positive A549 NSCLC cells (Figure 5b) inducing CEBPA upregulation upon treatment.

It was also found that CEBPA specific construct transfection induces a strong reduction of cell viability as expected by a CEBPA upregulating molecule (Figure 6).

The CaptaDiRs according to the invention is effective in the treatment of rare diseases associated to site-specific DNA methylation abnormalities, such as Fragile X Syndrome (FXS). The AptaDiR ability to restore FMR1 expression upon transfection of FXS fibroblasts (Figure 7) was demonstrated. Based on this encouraging data, captaDiR approach can be a promising platform to achieve FMR1 selective demethylation for the safe therapy of FXS.

From the above description and the above-noted examples, the advantage attained by the product described and obtained according to the present invention are apparent.

CLAIMS

1. A site-specific chimeric aptamer (captaDiR) capable of inhibiting DNA methyltransferase 1 (DNMT1) at a specific target gene locus, wherein the aptamer comprises:
- 5 - a first domain that is a oligonucleotide with a sequence deriving from aptaDiR that by assuming a stem loop structure RNA is capable of interacting with DNMT1; and
- a second domain that is either
- a gene-specific oligonucleotide with a sequence able to anchor the aptamer to
- 10 the specific target gene locus DNA sequence by forming an R loop with the DNA sequence of the target gene locus,
- or
- a gene-specific oligonucleotide with a sequence able to anchor the aptamer to the specific target gene locus DNA sequence by forming a triplex forming
- 15 sequence with the DNA sequence of the specific target gene locus;
- wherein said second domain is fused to the 5', or to the 3', of the stem loop structure RNA; and wherein said site-specific chimeric aptamer protects said target gene locus from DNMT1-mediated methylation, allowing the local activation of gene expression.
- 20
2. The site-specific chimeric aptamer according to claim 1, wherein the chimeric aptamer is capable of reducing DNMT1 function by at least about 10%, and/or the aptamer is capable of increasing the target gene expression level.
- 25
3. The site-specific chimeric aptamer according to claim 1 or 2, wherein the first domain of the chimeric aptamer has the sequence set forth in SEQ ID NO:33.
4. The site-specific chimeric aptamer according to any one of claims 1 to 3, wherein when the second domain forms a triplex forming sequence, it assumes

a triplex RNA-DNA helix structure by Reverse Hoogsteen base pairing with the DNA sequence of the specific target gene locus.

- 5 5. The site-specific chimeric aptamer according to any one of claims 1 to 4, said site-specific chimeric aptamer having a length in the range from 15 to 55 nucleotides, preferably in the range from 30 to 55 nucleotides, more preferably having a length of 52 nucleotides.
- 10 6. The site-specific chimeric aptamer according to any one of claims 1 to 5, said site-specific chimeric aptamer having a target gene chosen from the group consisting of: CEBPA (CCAAT enhancer binding protein alpha), SOCS3 (suppressor of cytokine signaling 3) and FMR1 (fragile X messenger ribonucleoprotein 1).
- 15 7. The site-specific chimeric aptamer according to any one of claims 1 to 6, said site-specific chimeric aptamer having any chemical and/or structural modifications, including a chimeric aptamer consisting of multiple copies of the aptamer domain fused to one or more targeting domains.
- 20 8. The site-specific chimeric aptamer according to any one of claims 1 to 7, said site-specific chimeric aptamer having the sequence set for in SEQ ID NO:46.
- 25 9. A pharmaceutical composition comprising one or more site-specific chimeric aptamers according to any one of the claims 1 to 8 and one or more pharmaceutically acceptable excipients.
- 30 10. A site-specific chimeric aptamer capable of inhibiting DNA methyltransferase 1 (DNMT1) at a specific target gene locus according to any one of claims 1 to 8 or a pharmaceutical composition according to claim 9, for use as a medicament.

11. A site-specific chimeric aptamer capable of inhibiting DNA methyltransferase 1 (DNMT1) at a specific target gene locus according to any one of claims 1 to 8 or a pharmaceutical composition according to claim 9, for use in the treatment of cancer, neurological, neurodegenerative, including Alzheimer's disease and
5 Alzheimer-like disorders, and rare diseases characterized by aberrant or altered DNA methylation.

12. The site-specific chimeric aptamer or the pharmaceutical composition for use according to claim 11, wherein said cancer is selected from the group
10 consisting of
optionally the disease characterized by aberrant DNA methylation is chronic myelogenous leukemia (CML), Myelodysplastic syndrome (MDS) and/or non-small cell lung cancer (NSCLC), including adenocarcinoma (such as human alveolar basal epithelial adenocarcinoma) and squamous cell carcinoma.

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