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(81) **Designated States** (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— with international search report (Art. 21(3))

— with sequence listing part of description (Rule 5.2(a))

NUCLEOLIN SPECIFIC APTAMER AND USE THEREOF

TECHNICAL FIELD

5 The present invention relates to improved G-rich oligonucleotide (GRO) aptamers specific to nucleolin, a method of preparing the aptamers and a use of the aptamers for diagnosing and/or treating a nucleolin-associated disease.

BACKGROUND

10 Nucleolin is a protein that is expressed at elevated levels in transformed cells. Tumor cells have been shown to present nucleolin on the cell surface as well as expressing it in the cytoplasm and nucleus. Nucleolin plays multiple roles in the cell and is involved in ribosome biogenesis, cell growth, and DNA replication.

 Aptamers are about 60~80mers of synthetic ribonucleic acid(RNA) or
15 deoxyribonucleic acid(DNA) oligonucleotides which are known to be discovered by the process called systematic evolution of ligands by exponential enrichment(SELEX) based on high affinity and specific molecular fit with their targets of interest.

 Aptamer have recently preferred to be applied for diagnosing and treating cancers as imaging target agents rather than monoclonal antibodies due to the following
20 characteristics: inexpensive, efficient and rapid for production, highly stable for long-term storage, versatile molecules that can be easily modified with imaging probe, small size (8-15 kDa) resulting low immune risk and better penetration into target tissues in vivo, and high affinity molecular probe.

 A large number of aptamers targeting cancer-related proteins, such as Wilim's
25 tumor protein 1(WT1), transcription factor 1(TCF-1), human epidermal growth factor receptor 3(HER-3), prostate-specific membrane antigene(PSMA), tenascin-C, nucleolin, pigpen and vascular endothelial growth factor(VEGF), have been developed to target and image cancers.

 Some chemical modifications on the ribose backbone of aptamer nucleotides
30 using 2'-amino or 2'-fluoro pyrimidines have been in situ and in vitro studied with the

existing aptamers to be resistant to nucleases, more capable of transfer across membranes or more capable of specific binding to the target of interest, but they influence the structure of aptamers, resulting in the loss of aptamer properties.

5 **SUMMARY OF THE INVENTION**

The present inventors found that chemically 5-modified deoxyuridine (dU)-containing GRO29A(SEQ ID NO: 1) and AS1411 (SEQ ID NO: 2) exhibit much higher affinity to nucleolin protein than that of non-modified AS1411 and GRO29A in various cancer cell types, to complete the present invention.

10 An embodiment provides a nucleolin-specific aptamer having the nucleotide sequence of SEQ ID NO: 1 or 2, wherein one or more thymidines (T) are independently substituted with a modified deoxyuridine (dU), and wherein the modified dU is a deoxyuridine having a hydrophobic group at 5' position. The modified deoxyuridine may be 5-(*N*-benzylcarboxyamide)-2'-deoxyuridine (BzdU), 5-(*N*-
15 naphthylcarboxyamide)-2'-deoxyuridine (NapdU), or 5-(*N*-4-pyrrolebenzylcarboxyamide)-2'-deoxyuridine (4-PBdU).

Another embodiment provides a pharmaceutical composition containing the nucleolin-specific aptamer. The pharmaceutical composition may a nucleolin inhibitor or agent for inhibiting an abnormal hyper-proliferation of cell, for example an
20 anticancer agent.

Another embodiment provides a method of diagnosing a hyper-proliferative cell disorder, such as cancer using the nucleolin-specific aptamer labeled with a detectable label.

Another embodiment provides a method of treating a nucleolin-associated
25 cancer using the nucleolin-specific aptamer.

Another embodiment provides a method of inhibiting an abnormal hyper-proliferation of cell using the nucleolin-specific aptamer.

Still another embodiment provides a method of inhibiting nucleolin using the nucleolin-specific aptamer.

30

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to improved G-rich oligonucleotide (GRO) aptamers specific to nucleolin, a method of preparing the aptamers and a use of the aptamers for diagnosing and/or treating a cancer.

5 GRO29A(TTTGGTGGTGGTGGTTGTGGTGGTGGTGG; SEQ ID NO: 1) and AS1411 (GGTGGTGGTGGTGGTTGTGGTGGTGGTGG; SEQ ID NO: 2) are G-rich oligonucleotide (GRO) aptamers comprising a single-strand DNA chain of 29 or 26 bases with unmodified phosphodiester linkages. G-rich oligonucleotide (GROs) are a class of non-antisense nucleic acids that exhibit potent anti-proliferative effects against almost every cancer cell type that was tested and thus, appears to have broad therapeutic potential. GRO29A and AS1411 have been known to bind to the nucleolin protein, which is expressed at elevated levels in transformed cells. Almost all tumor cells have been shown to present nucleolin on the cell surface as well as expressing it in the cytoplasm and nucleus. Nucleolin plays multiple roles in the cell and is involved in ribosome biogenesis, cell growth and DNA replication. The mechanism of GRO anti-proliferative activity appears to depend on their binding to the nucleolin protein.

GRO29A and AS1411 self-anneal to form a biomolecular quadruplex structure that is extremely stable and resistant to degradation by serum enzyme. GRO29A and AS1411 have shown activity against a wide range of solid and blood cancer cell lines in preclinical experiments and could therefore have potential against a variety of human cancers.

The inventors directly applied chemically modified pyrimidine-based nucleoside(s) (e.g., deoxyuridine (dU), deoxycytidine (dC), uridine (U), cytidine (C), etc.) into the GRO aptamers (AS1411 and GRO29A), which binds to nucleolin protein expressed in abnormally hyperproliferative cells, such as cancer cells, to find a type of GRO aptamer more capable of specific binding to abnormally hyperproliferative cells, such as cancer cells. The modified nucleoside may be a pyrimidine nucleoside modified by a hydrophobic group, such as benzyl group, a naphthyl group, or a pyrrolebenzyl group, at its 5' position. Modified nucleoside may be exemplified as 5-(N-benzylcarboxyamide)-2'-deoxyuridine(called BzdU), 5-(N-naphthylcarboxyamide)-2'-

deoxyuridine(called NapdU), 5-(*N*-4-pyrrolebenzylcarboxamide)-2'-deoxyuridine(called 4-PBdU), 5-(*N*-benzylcarboxamide)-2'-deoxycytidine (called BzdC), 5-(*N*-naphthylcarboxamide)-2'-deoxycytidine (called NapdC), 5-(*N*-4-pyrrolebenzylcarboxamide)-2'-deoxycytidine (called 4-PBdC), 5-(*N*-benzylcarboxamide)-2'-uridine(called BzU), 5-(*N*-naphthylcarboxamide)-2'-uridine(called NapU), 5-(*N*-4-pyrrolebenzylcarboxamide)-2'-uridine(called 4-PBU), 5-(*N*-benzylcarboxamide)-2'-cytidine (called BzC), 5-(*N*-naphthylcarboxamide)-2'-cytidine (called NapC), 5-(*N*-4-pyrrolebenzylcarboxamide)-2'-cytidine (called 4-PBC), and the like.

10 In the concrete embodiment, several hundred compounds of GRO aptamer (AS1411 and GRO29A)-containing modified dU such as BzdU, NapdU and 4-PBdU (BzdU-containing-, NapdU-containing- and 4-PBdU-containing GRO aptamer) were exemplarily synthesized by randomly substituting one to twelve thymidines(T) in GRO29A (SEQ ID NO: 1) and one to nine thymidine in AS1411 (SEQ ID NO: 2) with
 15 modified dUs. The modified pyrimidine nucleoside having a hydrophobic group, such as benzyl group, a naphthyl group, or a pyrrolebenzyl group, at its 5' position can be sufficiently exemplified by the modified dUs as described above. Several statistically quantified fluorescence measurement, qualified confocal imaging analysis, FACS analysis, and MTT assay demonstrated with replaced T by modified dUs of a
 20 particular position of GRO aptamers (AS1411 and GRO29A). The results show that the modified dU-containing GRO aptamers significantly increased the targeting affinity to various cell lines, implying that the position and number of substituents in GRO aptamers (AS1411 and GRO29A) are critical parameters to improve the aptamer function. In the present invention, it is revealed that chemical modification on the
 25 existing aptamers would enhance the binding and targeting affinity to targets of interest without additional SELEX procedure.

The inventors also found that AS1411, which is a modified form of GRO29A by deletion of 'TTT' present at 5' end of GRO29A, has similar or higher affinity to nucleolin compared to GRO29A, indicating that the three bases present at 5' end of
 30 GRO29A (SEQ ID NO: 1) play no important role in the affinity to nucleolin.

Therefore, the sequence and/or presence/absence of the three bases may not matter in the present invention, and thus following SEQ ID NO: 3 can also be included in the present invention:

NGGTGGTGGTGGTTGTGGTGGTGGTGGN (SEQ ID NO: 3)

5 wherein 'N' may be absent or 1 to 20 nucleosides, preferably 1 to 10 nucleosides, which is independently selected from the group consisting of adenosine(A), thymidine(T)/uridine(U), cytidine(C), and guanosine(G).

Hereinafter, the present invention is described in detail.

In an aspect, a nucleolin-specific aptamer is provided. As used herein,
10 'nucleolin-specific aptamer' means an aptamer having a specifically high affinity to nucleolin protein, thereby being capable of specifically binding to nucleolin protein.

The aptamer has the nucleotide sequence of SEQ ID NO: 3, preferably SEQ ID NO: 1 or 2, wherein one or more thymidines (T) are independently substituted with a modified pyrimidine nucleoside (e.g., deoxyuridine (dU), deoxycytidine (dC), uridine
15 (U), cytidine (C), etc.). The modified pyrimidine nucleoside may be a pyrimidine nucleoside having a hydrophobic group at 5' position. The hydrophobic group may have a benzyl group, a naphthyl group, or a pyrrolebenzyl group. By such modification of pyrimidine nucleoside with a hydrophobic group, the affinity of the aptamer to nucleolin is considerably improved compared with that of non-modified
20 aptamer.

In a concrete embodiment, the hydrophobic group may be benzylcarboxyamide, naphthylcarboxyamide, pyrrolebenzylcarboxyamide and the like, and accordingly, the modified pyrimidine nucleoside may be 5-(*N*-benzylcarboxyamide)-2'-deoxyuridine(called BzdU), 5-(*N*-naphthylcarboxyamide)-2'-deoxyuridine(called
25 NapdU), 5-(*N*-4-pyrrolebenzylcarboxyamide)-2'-deoxyuridine(called 4-PBdU), 5-(*N*-benzylcarboxyamide)-2'- deoxycytidine (called BzdC), 5-(*N*-naphthylcarboxyamide)-2'-deoxycytidine (called NapdC), 5-(*N*-4-pyrrolebenzylcarboxyamide)-2'-deoxycytidine (called 4-PBdC), 5-(*N*-benzylcarboxyamide)-2'-uridine(called BzU), 5-(*N*-naphthylcarboxyamide)-2'-uridine(called
30 NapU), 5-(*N*-4-pyrrolebenzylcarboxyamide)-2'-uridine(called 4-PBU), 5-(*N*-benzylcarboxyamide)-2'-

cytidine (called BzC), 5-(*N*-naphthylcarboxamide)-2'-cytidine (called NapC), 5-(*N*-4-pyrrolebenzylcarboxamide)-2'-cytidine (called 4-PBC), and the like.

The inventors found that the position of thymidine to be modified is also important to improve the affinity to nucleolin. The modification of thymidines present at central region of the aptamer considerably contributes to improve the affinity to nucleolin. The central region may be a loop site of the aptamer. More specifically, the central region may be 12th to 18th positions, preferably 15th and 16th positions of SEQ ID NO: 1, or 9th to 18th positions, preferably 12th and 13th positions of SEQ ID NO: 2 or SEQ ID NO: 3 (when the positions are counted starting from 'G' after 'N' at 5'-end). As shown in Fig. 23, it is found that a more modification on any position in addition to central double modified dU-containing GRO29A or AS1411 did not increase binding to nucleolin on various cancer cell lines, indicating that the central region (2 bases) of the aptamer may be a critical region for the modification of the aptamer to effect on the affinity of the aptamer to nucleolin.

Therefore, in a preferable embodiment, at least two thymidines present in 12th to 18th positions, preferably at 15th and 16th positions of SEQ ID NO: 1, or present in 9th to 18th positions, preferably at 12th and 13th positions of SEQ ID NO: 2 or SEQ ID NO: 3 (when the positions are counted starting from 'G' after 'N' at 5'-end) are substituted with the modified deoxyuridine. In a concrete embodiment, the aptamer has the nucleotide sequence of SEQ ID NO: 1, wherein 2 to 12 thymidines essentially comprising two thymidines present in 12th to 18th positions, preferably at 15th and 16th positions are replaced with modified deoxyuridines. In another concrete embodiment, the aptamer has the nucleotide sequence of SEQ ID NO: 2 or SEQ ID NO: 3 (when the positions are counted starting from 'G' after 'N' at 5'-end), wherein 2 to 9 thymidines essentially comprising two thymidines present in 9th to 18th positions, preferably at 12th and 13th positions are replaced with modified deoxyuridines.

In another aspect, a method of preparing the nucleolin-specific aptamer according to the present invention is provided. The method may comprise the steps of replacing one or more thymidines (T) present in the nucleotide sequence of SEQ ID NO: 1 or 2 with the modified pyrimidine nucleoside(s) (e.g., dU, dC, U, C, etc.) as

described above.

Nucleolin functions as a marker of hyper-proliferative cells, such as cancer cells, since nucleolin is specifically expressed on surface of hyper-proliferative cells, such as cancer cells. Therefore, the nucleolin-specific aptamer according to the present
5 invention can be useful in diagnosing various hyper-proliferative cell disorders.

As used herein, the hyper-proliferative cell disorder refers to excess (abnormally high) cell proliferation (abnormal hyper-proliferation of cell), relative to that occurring with the same type of cell in the general population and/or the same type of cell obtained from a patient at an earlier time. The term denotes malignant as well
10 as non-malignant cell populations. Such disorders have an excess cell proliferation of one or more subsets of cells, which often appear to differ from the surrounding tissue both morphologically and genotypically. The excess cell proliferation can be determined by reference to the general population and/or by reference to a particular patient, e.g. at an earlier point in the individual's life. Hyper-proliferative cell
15 disorders can occur in different types of animals and in humans, and produce different physical manifestations depending upon the affected cells. The hyper-proliferative cell disorders may include various cancers.

Therefore, in another aspect, a composition for diagnosing a hyper-proliferative cell disorder, such as a cancer, containing the nucleolin-specific aptamer according to
20 the present invention as an active ingredient, and a method of diagnosing a hyper-proliferative cell disorder, such as a cancer, using the nucleolin-specific aptamer, are provided. Alternatively, a use of the nucleolin-specific aptamer according to the present invention in diagnosing a hyper-proliferative cell disorder is provided. The method may comprise the steps of:

25 contacting the nucleolin-specific aptamer with a sample from a subject, wherein the aptamer is labeled with a detectable label; and
 detecting a signal from the label.

In the method, the subject is determined as having a hyper-proliferative cell disorder, such as a cancer, when the signal is detected. The nucleolin specific aptamer
30 is as described above.

The subject to be diagnosed may be from any mammalian species, e.g. primate sp., particularly humans; rodents including mice, rats and hamsters; rabbits; equines, bovines, canines, felines; and the like. Animal models may be of interest for experimental investigations, providing a model for treatment of human disease. The sample may be any bio-sample from the subject, such as cells, tissues, blood, body fluid, and the like.

The cancer that can be diagnosed by the present invention may be any nucleolin-associated cancer including any solid cancers and blood cancers, including leukemias, lymphomas (Hodgkins and non-Hodgkins), and other myeloproliferative disorders; carcinomas of solid tissue, sarcomas, melanomas, adenomas, hypoxic tumors, squamous cell carcinomas of the mouth, throat, larynx, or lung, genitourinary cancers such as cervical and bladder cancer, hematopoietic cancers, head and neck cancers, and nervous system cancers, benign lesions such as papillomas, and the like. The nucleolin-associated cancer may be selected from the group consisting of leukemia, lymphoma, breast cancer, liver cancer, gastric cancer, ovarian carcinoma, cervical carcinoma, glioma cancer, colon cancer, lung cancer, pancreas cancer, prostate cancer, liver cancer, stomach cancer, uterine cancer, bladder cancer, thyroid cancer, ovary cancer, melanoma cancer, cervical cancer, and the like, but not be limited thereto.

The label may be any one which can be detectable by any conventional means. For example, the label may be one or more selected from the group consisting of a fluorescence material, infrared material, quantum dots, ion oxide bead, PET probe (e.g., $^{68}\text{gallium}$), T1 MR probe including iron oxide (e.g., Fe_3O_4), T2 MR probe (e.g., MnFe_2O_4 , or GdFe_2O_4 nanoparticles), and the like, but not be limited thereto.

When the labeled-nucleolin specific aptamer is contacted with the sample, and then, non-reacted aptamer is removed (for example, by washing), if nucleolin is present in the sample (i.e., the subject having abnormally hyper-proliferative cells, such as cancer cells, resulted from the presence of nucleolin), the aptamer specifically binds to nucleolin on cells, and the signal from the label attached to the aptamer is detected, allowing to diagnose a hyper-proliferative cell disease, such as a cancer, as described above.

Nucleolin is associated with cell cycle and cell division, and thus, when the nucleolin specific aptamer of the present invention binds to nucleolin, thereby interfering with the function of nucleolin, resulting in interfering with the cell cycle, arresting cell-cycle, for example at the S-phase, inhibiting DNA replication, inducing
5 cell death, etc. Therefore, the nucleolin specific aptamer of the present invention can function as an inhibitor of nucleolin, and agent for inhibiting hyperproliferation of cell, and thereby being useful in treating a hyper-proliferative cell disease, such as cancer, as described above.

Therefore, in another aspect, a composition for inhibiting nucleolin, containing
10 the nucleolin-specific aptamer as an active ingredient, and a method of inhibiting nucleolin using the nucleolin-specific aptamer, are provided. Alternatively, a use of the nucleolin-specific aptamer in inhibiting nucleolin is provided. The method may comprise the step of administering the nucleolin-specific aptamer according to the present invention to a subject or a sample comprising nucleolin-expressing cells. In
15 addition, a method of inhibiting hyperproliferation of cell caused by nucleolin using the nucleolin-specific aptamer is also provided. The method may comprise the step of administering the nucleolin-specific aptamer of Claim 1 to a subject or a sample comprising nucleolin-expressing cells.

The nucleolin-specific aptamer is as described above. The subject may be
20 from any mammalian species, e.g. primate sp., particularly humans; rodents including mice, rats and hamsters; rabbits; equines, bovines, canines, felines; and the like, who is in need of the inhibition of nucleolin and/or hyperpriliferation of cell caused by nucleolin. Animal models may be of interest for experimental investigations, providing a model for treatment of human disease. The sample may be any bio-sample
25 from the subject, such as cells, tissues, blood, body fluid, and the like.

In another aspect, a composition for inhibiting hyperproliferation of cell caused by nucleolin, containing the nucleolin-specific aptamer as an active ingredient, and a method of inhibiting an abnormal hyper-proliferation of cell using the nucleolin-specific aptamer according to the present invention, are provided. Alternatively, a use of the
30 nucleolin-specific aptamer in inhibiting hyperproliferation of cell caused by nucleolin is

provided. In addition, a composition, a use, and a method of treating a hyper-proliferative cell disorder, such as a cancer (nucleolin-associated cancer), using the nucleolin-specific aptamer according to the present invention, are also provided.

5 The method may comprise the step of administering an effective amount of the nucleolin-specific aptamer to a subject who needs the inhibition of the abnormal hyper-proliferation of cell and/or the treatment of the abnormal hyper-proliferation of cell, for example, the treatment of a cancer. As described above, the nucleolin-specific aptamer has an excellent affinity to nucleolin to inhibit nucleolin, thereby exhibiting a treatment effect for cell hyper-proliferation, such as a cancer.

10 The nucleolin specific aptamer is as described above. The subject may be from any mammalian species, e.g. primate sp., particularly humans; rodents including mice, rats and hamsters; rabbits; equines, bovines, canines, felines; and the like, who needs the inhibition of the abnormal hyper-proliferation of cell and/or the treatment of the abnormal hyper-proliferation of cell, for example, the treatment of a cancer. Animal
15 models may be of interest for experimental investigations, providing a model for treatment of human disease. The cancer that can be treated by the present invention may be any nucleolin-associated cancer including any solid cancers and blood cancers, including leukemias, lymphomas (Hodgkins and non-Hodgkins), and other myeloproliferative disorders; carcinomas of solid tissue, sarcomas, melanomas, adenomas, hypoxic tumors, squamous cell carcinomas of the mouth, throat, larynx, and
20 lung, genitourinary cancers such as cervical and bladder cancer, hematopoietic cancers, head and neck cancers, and nervous system cancers, benign lesions such as papillomas, and the like. The nucleolin-associated cancer may be selected from the group consisting of leukemia, lymphoma, breast cancer, liver cancer, gastric cancer, ovarian carcinoma, cervical carcinoma, glioma cancer, colon cancer, lung cancer, pancreas
25 cancer, prostate cancer, liver cancer, stomach cancer, uterine cancer, bladder cancer, thyroid cancer, ovary cancer, melanoma cancer, cervical cancer, and the like, but not be limited thereto.

The effective amount means an amount exhibiting a therapeutic effect on the
30 inhibition of nucleolin or hyper-proliferation of cell, for example treating a cancer, and

may be properly controlled depending on the condition of the subject and/or severity of disease. The effective amount can be administered in one or more administrations. The administration may be performed by oral or parenteral (e.g., intravenous, subcutaneous, intramuscular, and the like) pathway, but not limited thereto.

5 In still another aspect, a pharmaceutical composition containing the nucleolin-specific aptamer according to the present invention as an active ingredient is provided. The pharmaceutical composition may a nucleolin inhibitor or agent for inhibiting an abnormal hyper-proliferation of cell, for example an anticancer agent.

10 **BRIEF DESCRIPTION OF THE FIGURES**

Fig.1 shows a schematic diagram to synthesize nucleolin aptamer containing 5-(N-benzylcarboxyamide)-2'-deoxyuridine (BzdU-containing GRO29A), wherein Z indicates that thymidines in GRO29A oligonucleotides were substituted with 5-(N-benzylcarboxyamide)-2'-deoxyuridine(BzdU).

15 Fig. 2 shows results of fluorescence analysis of Cy3-labeled BzdU-containing GRO29A compounds targeting C6 cells.

Fig. 3 shows confocal microscopy images of Cy3-labeled BzdU-containing GRO29A targeting C6 cells.

Fig. 4 shows fluorescence intensities measured by fluorescence analysis of numbers 1642-39, 1642-51, 1642-19 and Cy3-labeled GRO29A, and Cy3-labeled CRO29A (the control form of GRO29A labeled with Cy3) in HeLa and CHO cells.

Figs. 5A and 5B show confocal microscopy images in HeLa cells (5A) and CHO cells (5B).

Fig. 6 shows cell viabilities measured by MTT assay to show anti-proliferation effects.

Fig. 7 shows results of fluorescence analysis of Cy3-labeled NapdU-containing GRO29A compounds targeting C6 cells.

Fig. 8 shows results of a flow cytometric analysis of MDA-MB231 cells treated with NapdU -containing AS1411.

30 Fig. 9 shows results of a flow cytometric analysis of MDA-MB231 cells treated

with 4-PBdU -containing AS1411.

Fig. 10 shows results of a flow cytometric analysis of HepG2 cells treated with NapdU -containing AS1411.

Fig. 11 shows results of a flow cytometric analysis of HepG2 cells treated with
5 4-PBdU -containing AS1411.

Fig. 12 shows results of a flow cytometric analysis of AGS cells treated with NapdU -containing AS1411.

Fig. 13 shows results of a flow cytometric analysis of AGS cells treated with 4-PBdU -containing AS1411.

Fig. 14 shows results of a flow cytometric analysis of OVCAR-3 cells treated
10 with NapdU -containing AS1411.

Fig. 15 shows results of a flow cytometric analysis of OVCAR-3 cells treated with 4-PBdU -containing AS1411.

Fig. 16 shows results of a flow cytometric analysis of HeLa cells treated with
15 NapdU -containing AS1411.

Fig. 17 shows results of a flow cytometric analysis of HeLa cells treated with 4-PBdU -containing AS1411.

Fig. 18 shows results of a flow cytometric analysis of U87MG cells treated with NapdU -containing AS1411.

Fig. 19 shows results of a flow cytometric analysis of U87MG cells treated with
20 4-PBdU -containing AS1411.

Fig. 20 shows results of a flow cytometric analysis of NIHT3 cells treated with NapdU -containing AS1411.

Fig. 21 shows results of a flow cytometric analysis of NIHT3 cells treated with
25 4-PBdU -containing AS1411.

Fig. 22 shows results of a quantification of FACS analysis for various cell lines.

Fig. 23 shows activities between central double modification and a more modification of AS1411.

Fig. 24 shows specificities of all NapdU-containing AS1411(1642-132) and
30 central double NapdU-containing AS1411(1642-161) in cancer and normal cell lines

determined with FACS analysis.

Fig. 25 shows specificities of all 4-PBdU-containing AS1411(1642-206) and central double 4-PBdU-containing AS1411(1642-177) in cancer and normal cell lines determined with FACS analysis.

5 Fig. 26 shows results of a quantification of FACS analysis data of AS1411 and central modified dU-containing AS1411 binding to nucleolin on various cell lines

Fig. 27 shows MR images of tumor-bearing mice before and after tail-vein injection of central double NapdU-containing AS1411(1642-161), wherein dark signal intensities at tumor sites were detected in AS1411-MF particle- and modified AS1411-MF-injected mice (arrowhead).
10

Fig. 28 shows the effect of central double NapdU-containing AS1411 on the cell viability of MDA-MB231 breast cancer cells.

Fig. 29 shows the relation between structure and activity of central double modified dU-containing AS1411 (or GRO29A).
15

EXAMPLES

A better understanding of the present invention may be obtained in light of the following examples that are set forth to illustrate, but are not to be construed to limit, the present invention.

20

EXAMPLE 1: Preparation of Cy3-labeled modified dU AS1411 and GRO29A

1.1: Design of Cy3-labeled modified dU AS1411 and GRO29A

Forty-seven different compounds of Cy3-labeled BzdU-containing GRO29A
25 were designed and synthesized. The GRO29A oligonucleotides (TTTGGTGGTGGTGGTTGTGGTGGTGGTGG, SEQ ID NO: 1) incorporated with 5-(*N*-benzylcarboxyamide)-2'-deoxyuridine(BzdU) and labeled with Cy3 were prepared according to the following synthesis procedure of Example 1.2. One to twelve thymidines in GRO29A oligonucleotides were randomly replaced with BzdU (see Fig.
30 1). Fig. 1 is a schematic diagram to synthesize nucleolin aptamer containing 5-(*N*-

benzylcarboxamide)-2'-deoxyuridine (BzdU-containing GRO29A), wherein Z indicated where thymidines in GRO29A oligonucleotides were substituted with 5-(*N*-benzylcarboxamide)-2'-deoxyuridine(BzdU).

The designed forty-seven Cy3-labeled BzdU-containing GRO29A are summarized in Table 1.

(Table 1) A list of modified GRO29A containing Bz at 5 position of dU (5-(*N*-benzylcarboxamide)-2'-deoxyuridine, BzdU)

Comp. No.	Cy3-labeled Modified dU- GRO29A Sequence (5'→3')	Cal. MS	Obs. MS
Cy3-1642-8	Cy3-labeled-ZTTGGTGGTGGTGGTTGTGGTGGTGGTGG	9811.71	9812.32
Cy3-1642-9	Cy3-labeled-TZTGGTGGTGGTGGTTGTGGTGGTGGTGG	9811.71	9811.72
Cy3-1642-10	Cy3-labeled-TTZGGTGGTGGTGGTTGTGGTGGTGGTGG	9811.71	9811.92
Cy3-1642-11	Cy3-labeled-TTTGGZGGTGGTGGTTGTGGTGGTGGTGG	9811.71	9812.27
Cy3-1642-12	Cy3-labeled-TTTGGTGGZGGTGGTTGTGGTGGTGGTGG	9811.71	9811.83
Cy3-1642-13	Cy3-labeled-TTTGGTGGTGGZGGTTGTGGTGGTGGTGG	9811.71	9812.38
Cy3-1642-14	Cy3-labeled-TTTGGTGGTGGTGGZTGTGGTGGTGGTGG	9811.71	9812.18
Cy3-1642-15	Cy3-labeled-TTTGGTGGTGGTGGTZGTGGTGGTGGTGG	9811.71	9812.22
Cy3-1642-16	Cy3-labeled-TTTGGTGGTGGTGGTTGZGGTGGTGGTGG	9811.71	9812.01
Cy3-1642-17	Cy3-labeled-TTTGGTGGTGGTGGTTGTGGZGGTGGTGG	9811.71	9812.10
Cy3-1642-18	Cy3-labeled-TTTGGTGGTGGTGGTTGTGGTGGZGGTGG	9811.71	9812.69
Cy3-1642-19	Cy3-labeled-TTTGGTGGTGGTGGTTGTGGTGGTGGZGG	9811.71	9811.78
Cy3-1642-21	Cy3-labeled-TZZGGTGGTGGTGGTTGTGGTGGTGGTGG	9930.82	9931.40
Cy3-1642-23	Cy3-labeled-ZZZGGTGGTGGTGGTTGTGGTGGTGGTGG	9930.82	9931.24
Cy3-1642-24	Cy3-labeled-TTTGGTGGTGGTGGTTGTGGTGGZGGZGG	9930.82	9931.02
Cy3-1642-25	Cy3-labeled-TTTGGTGGTGGTGGTTGTGGZGGTGGZGG	9930.82	9931.10
Cy3-1642-26	Cy3-labeled-TTTGGTGGTGGTGGTTGZGGTGGTGGZGG	9930.82	9931.81
Cy3-1642-27	Cy3-labeled-TTTGGTGGTGGTGGTZGTGGTGGTGGZGG	9930.82	9930.93
Cy3-1642-28	Cy3-labeled-TTTGGTGGTGGTGGZTGTGGTGGTGGZGG	9930.82	
Cy3-1642-29	Cy3-labeled-TTTGGTGGTGGZGGTTGTGGTGGTGGZGG	9930.82	
Cy3-1642-30	Cy3-labeled-TTTGGTGGZGGTGGTTGTGGTGGTGGZGG	9930.82	
Cy3-1642-31	Cy3-labeled-TTTGGZGGTGGTGGTTGTGGTGGTGGZGG	9930.82	9931.81

Cy3-1642-32	Cy3- labeled-TT Z GGTGGTGGTGGTTGTGGTGGTGG Z GG	9930.82	
Cy3-1642-33	Cy3- labeled-T Z TGGTGGTGGTGGTTGTGGTGGTGG Z GG	9930.82	
Cy3-1642-34	Cy3- labeled- Z TTGGTGGTGGTGGTTGTGGTGGTGG Z GG	9930.82	
Cy3-1642-35	Cy3- labeled-TTTGGTGGTGGTGG ZZ GTGGTGGTGG Z GG	10049.93	
Cy3-1642-36	Cy3- labeled-T ZZ GGTGGTGGTGGTTGTGGTGGTGG Z GG	10049.93	
Cy3-1642-37	Cy3- labeled- ZZ TGGTGGTGGTGGTTGTGGTGGTGG Z GG	10049.93	10051.40
Cy3-1642-39	Cy3- labeled-TTTGGTGGTGGTGG ZZ GTGGTGGTGGTGG	9930.82	
Cy3-1642-40	Cy3- labeled-TTTGGTGGTGGTGG ZZGZ GGTGGTGGTGG	10049.93	
Cy3-1642-41	Cy3- labeled-TTTGGTGGTGGTGG ZZGTGGZ GGTGGTGG	10049.93	
Cy3-1642-42	Cy3- labeled-TTTGGTGGTGGTGG ZZGTGGTGGZ GGTGG	10049.93	
Cy3-1642-43	Cy3- labeled-TTTGGTGGTGG ZGGZZ GTGGTGGTGGTGG	10049.93	
Cy3-1642-44	Cy3- labeled-TTTGGTGG ZGGTGGZZ GTGGTGGTGGTGG	10049.93	
Cy3-1642-45	Cy3- labeled-TTTGG ZGGTGGTGGZZ GTGGTGGTGGTGG	10049.93	10050.99
Cy3-1642-46	Cy3- labeled-TT Z GGTGGTGGTGG ZZ GTGGTGGTGGTGG	10049.93	
Cy3-1642-47	Cy3- labeled-T Z TGGTGGTGGTGG ZZ GTGGTGGTGGTGG	10049.93	
Cy3-1642-48	Cy3- labeled- Z TTGGTGGTGGTGG ZZ GTGGTGGTGGTGG	10049.93	
Cy3-1642-49	Cy3- labeled-T ZZ GGTGGTGGTGG ZZ GTGGTGGTGGTGG	10169.04	
Cy3-1642-50	Cy3- labeled- ZZ TGGTGGTGGTGG ZZ GTGGTGGTGGTGG	10169.04	
Cy3-1642-51	Cy3- labeled- ZT ZGGTGGTGGTGG ZZ GTGGTGGTGGTGG	10169.04	
Cy3-1642-52	Cy3- labeled- ZZZ GGTGGTGGTGG ZZ GTGGTGGTGGTGG	10288.15	10288.41
Cy3-1642-53	Cy3- labeled-TTTGGTGGTGG ZGGTTGZ GGTGGTGGTGG	9930.82	
Cy3-1642-54	Cy3- labeled-TTTGGTGG ZGGTGGTTGTGGZ GGTGGTGG	9930.82	
Cy3-1642-55	Cy3- labeled-TTTGG ZGGTGGTGGTTGTGGTGGZ GGTGG	9930.82	
Cy3-1642-56	Cy3- labeled-TTTGGTGG ZGGZGGTTGZGGZGGZ GGTGG	10288.15	
Cy3-1642-57	Cy3- labeled-TTTGG ZGGZGGZGGTTGZGGZ GGTGG Z GG	10407.26	
Cy3-1641-3(GRO29A)	Cy3- labeled-TTTGGTGGTGGTGGTTGTGGTGGTGGTGG	9692.6 0	9693.85
Cy3-1641-8(CRO29A)	Cy3- labeled-TTTCCTCCTCCTCCTTCTCCTCCTCCTCC	9012.09	9012.70

Z: BzdU

Cy3-labeled BzdU-containing GRO29A derivatives contained 5-(*N*-benzylcarboxamide)-2'-deoxyuridine(BzdU) in Z. CRO29A indicated the control form of GRO29A, wherein all 'G's in GRO29A are substituted with 'C'.

- 5 The GRO29A oligonucleotides incorporated with 5-(*N*-naphthylcarboxamide)-2'-deoxyuridine(NapdU) instead of BzdU and labeled with Cy3 were also designed (see Table 2), and prepared according to the following synthesis procedure of Example 1.2.

(Table 2) A list of modified GRO29A containing Nap at 5 position of dU (5-(*N*-naphthylcarboxamide)-2'-deoxyuridine, NapdU)

Comp. No.	Cy3-labeled Modified dU- GRO29A Sequence (5'→3')	MS
1642-59	TTTGG Z GGTGGTGGTTGTGGTGGTGGTGG	9861.77
1642-60'	TTTGGTGG Z GGTGGTTGTGGTGGTGGTGG	9861.77
1642-61	TTTGGTGGTGG Z GGTTGTGGTGGTGGTGG	9861.77
1642-62	TTTGGTGGTGGTGGTTGTGGTGGTGG Z GG	9861.77
1642-63	TTTGGTGG Z GGTGGTTGTGGTGGTGG Z GG	10030.94
1642-64	TTTGG Z GGTGGTGGTTGTGGTGGTGG Z GG	10030.94
1642-65	Z ZTGGTGGTGGTGGTTGTGGTGGTGG Z GG	10220.11
1642-66	TTTGGTGGTGGTGG Z ZGTGGTGGTGGTGG	10030.94
1642-68	TTTGGTGGTGGTGG Z ZGGTGGTGGTGG	10200.11
1642-67	TTTGGTGGTGGTGG Z ZGTGG Z GGTGGTGG	10200.11
1642-69	T Z TGGTGGTGGTGG Z ZGTGGTGGTGGTGG	10200.11
1642-70	Z TTGGTGGTGGTGG Z ZGTGGTGGTGGTGG	10200.11
1642-71	T Z ZGGTGGTGGTGG Z ZGTGGTGGTGGTGG	10369.28
1642-72	Z TZGGTGGTGGTGG Z ZGTGGTGGTGGTGG	10369.28
1642-73	Z ZZGGTGGTGGTGG Z ZGTGGTGGTGGTGG	10538.45
1642-74	TTTGG Z GG Z GG Z GGTTG Z GG Z GGTGG Z GG	10707.62
GRO29A	TTTGGTGGTGGTGGTTGTGGTGGTGGTGG	9692.6
CRO29A	TTTCCTCCTCCTCCTTCTCCTCCTCCTCC	9012.09

Cy3-labeled NapdU-containing GRO29A derivatives contained 5-(*N*-naphthylcarboxyamide)-2'-deoxyuridine (NapdU) in Z. CRO29A indicated the control form of GRO29A, wherein all 'G's in GRO29A are substituted with 'C'.

- 5 Modified dU-containing AS1411 derivatives were also designed and synthesized by randomly substituting one to nine thymidine(T) in AS1411(GGTGGTGGTGGTTGTGGTGGTGGTGG, SEQ ID NO: 2). Modified dU inserting on AS1411 is independently selected from 5-(*N*-benzylcorboxyamide)-2'-deoxyuridine[BzdU] and 5-(*N*-naphthylcarboxyamide)-2'-deoxyuridine[NapdU] and 5-
10 (*N*-4-pyrrimidylbenzylcarboxyamide)-2'-deoxyuridine[4-PBdU]. The modified dU-containing AS1411 derivatives were summarized in Tables 3-5.

(Table 3) A list of modified AS1411 having hydrophobic groups such as Bz at 5 position of dU (5-(*N*-benzylcarboxyamide)-2'-deoxyuridine, BzdU)

Comp. No.	Cy3-labeled Modified dU-AS1411 sequence	MS
Cy3-1642-88	Cy3-GGZGGZGGZGGZZGZGGZGGZGGZGG	9851.99
Cy3-1642-89	Cy3-GGZGGZGGZGGTTGTGGZGGZGGZGG	9494.66
Cy3-1642-90	Cy3-GGZGGZGGTGGTTGTGGTGGZGGZGG	9256.44
Cy3-1642-91	Cy3-GGTGGZGGZGGTTGTGGZGGZGGTGG	9256.44
Cy3-1642-92	Cy3-GGZGGTGGTGGTTGTGGTGGTGGZGG	9018.22
Cy3-1642-93	Cy3-GGTGGZGGTGGTTGTGGTGGZGGTGG	9018.22
Cy3-1642-94	Cy3-GGTGGTGGZGGTTGTGGZGGTGGTGG	9018.22
Cy3-1642-95	Cy3-GGZGGTGGZGGTTGTGGZGGTGGZGG	9256.44
Cy3-1642-96	Cy3-GGTGGTGGTGGTZZGTGGTGGTGGTGG	8899.11
Cy3-1642-97	Cy3-GGTGGTGGTGGZZGZGGTGGTGGTGG	9137.33
Cy3-1642-98	Cy3-GGTGGTGGTGGZTGZGGTGGTGGTGG	9018.22
Cy3-1642-82	Cy3-GGZGGTGGTGGTTGTGGTGGTGGTGG	8899.11
Cy3-1642-99	Cy3-GGTGGZGGTGGTTGTGGTGGTGGTGG	8899.11

Cy3-1642-100	Cy3-GGTGGTGGZGGTTGTGGTGGTGGTGG	8899.11
Cy3-1642-101	Cy3-GGTGGTGGTGGZTGTGGTGGTGGTGG	8899.11
Cy3-1642-102	Cy3-GGTGGTGGTGGTGTGGTGGTGGTGG	8899.11
Cy3-1642-103	Cy3-GGTGGTGGTGGTTGTGGTGGTGGTGG	8899.11
Cy3-1642-104	Cy3-GGTGGTGGTGGTTGTGGZGGTGGTGG	8899.11
Cy3-1642-105	Cy3-GGTGGTGGTGGTTGTGGTGGZGGTGG	8899.11
Cy3-1642-83	Cy3-GGTGGTGGTGGTTGTGGTGGTGGZGG	8899.11
Cy3-1642-106	Cy3-GGTGGTGGTGGTTGTGGTGGZGGZGG	9018.22
Cy3-1642-107	Cy3-GGTGGTGGTGGTTGTGGZGGTGGZGG	9018.22
Cy3-1642-108	Cy3-GGTGGTGGTGGTTGTGGTGGTGGZGG	9018.22
Cy3-1642-109	Cy3-GGTGGTGGTGGTGTGGTGGTGGZGG	9018.22
Cy3-1642-110	Cy3-GGTGGTGGTGGZTGTGGTGGTGGZGG	9018.22
Cy3-1642-111	Cy3-GGTGGTGGZGGTTGTGGTGGTGGZGG	9018.22
Cy3-1642-112	Cy3-GGTGGZGGTGGTTGTGGTGGTGGZGG	9018.22
Cy3-1642-113	Cy3-GGZGGTGGTGGTTGTGGTGGTGGZGG	9018.22
Cy3-1642-114	Cy3-GGTGGTGGTGGZZGTGGTGGTGGZGG	9137.33
Cy3-1642-115	Cy3-GGTGGTGGTGGZZGTGGTGGTGGTGG	9018.22
Cy3-1642-116	Cy3-GGTGGTGGTGGZZGZGGTGGTGGTGG	9137.33
Cy3-1642-117	Cy3-GGTGGTGGTGGZZGTGGZGGTGGTGG	9137.33
Cy3-1642-118	Cy3-GGTGGTGGTGGZZGTGGTGGZGGTGG	9137.33
Cy3-1642-119	Cy3-GGTGGTGGZGGZZGTGGTGGTGGTGG	9137.33
Cy3-1642-120	Cy3-GGTGGZGGTGGZZGTGGTGGTGGTGG	9137.33
Cy3-1642-121	Cy3-GGZGGTGGTGGZZGTGGTGGTGGTGG	9137.33
Cy3-1642-122	Cy3-GGTGGTGGZGGTTGTGGTGGTGGTGG	9018.22
Cy3-1642-123	Cy3-GGTGGZGGTGGTTGTGGZGGTGGTGG	9018.22
Cy3-1642-124	Cy3-GGZGGTGGTGGTTGTGGTGGZGGTGG	9018.22
Cy3-1642-125	Cy3-GGTGGZGGZGGTTGTGGZGGZGGTGG	9375.55
Cy3-1642-126	Cy3-GGZGGZGGZGGTTGTGGZGGTGGZGG	9494.66
Cy3-1642-127	Cy3-ZGGTGGTGGTGGTTGTGGTGGTGGTGGZ	9626.62

Cy3-1642-129	Cy3-CC Z CC Z CC Z CC Z Z Z CC Z CC Z CC Z CC	9171.48
Cy3-1642-130	Cy3-CC Z CC Z CC Z CC T TCTCC Z CC Z CC Z CC	8814.15
Cy3-1642-131	Cy3-CCTCCTCCTCC Z Z Z CCTCCTCCTCC	8456.82
Cy3-1642-80 (AS1411)	Cy3-GGTGGTGGTGGTTGTGGTGGTGGTGG	8780.0
Cy3-1642-81 (Control AS1411)	Cy3-CCTCCTCCTCCTTCTCCTCCTCCTCC	8099.49

Z = BzdU

Cy3-labeled BzdU-containing AS1411 derivatives contained 5-(*N*-benzylcarboxyamido)-2'-deoxyuridine (BzdU) in Z. Control AS1411 indicated the control form of AS1411, wherein all 'G's in AS1411 are substituted with 'C'..

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(Table 4) A list of modified AS1411 having hydrophobic groups such as Nap at 5 position of dU (5-(*N*-naphthylcarboxyamido)-2'-deoxyuridine, NapdU)

Comp. No.	Cy3-labeled Modified dU-AS1411 sequence	MS
Cy3-1642-132	Cy3-GG Z GG Z GG Z GG Z Z Z GG Z GG Z GG Z GG	10302.53
Cy3-1642-133	Cy3-GG Z GG Z GG Z GGTTGTGG Z GG Z GG Z GG	9795.02
Cy3-1642-134	Cy3-GG Z GG Z GGTGGTTGTGGTGG Z GG Z GG	9456.68
Cy3-1642-135	Cy3-GGTGG Z GG Z GGTTGTGG Z GG Z GGTGG	9456.68
Cy3-1642-136	Cy3-GG Z GGTGGTGGTTGTGGTGGTGG Z GG	9118.34
Cy3-1642-137	Cy3-GGTGG Z GGTGGTTGTGGTGG Z GGTGG	9118.34
Cy3-1642-138	Cy3-GGTGGTGG Z GGTTGTGG Z GGTGGTGG	9118.34
Cy3-1642-139	Cy3-GG Z GGTGG Z GGTTGTGG Z GGTGG Z GG	9456.68
Cy3-1642-140	Cy3-GGTGGTGGTGGT Z GTGGTGGTGGTGG	8949.17
Cy3-1642-141	Cy3-GGTGGTGGTGG Z Z Z GGTGGTGGTGG	9287.51
Cy3-1642-142	Cy3-GGTGGTGGTGG Z TG Z GGTGGTGGTGG	9118.34
Cy3-1642-143	Cy3-GG Z GGTGGTGGTTGTGGTGGTGGTGG	8949.17
Cy3-1642-144	Cy3-GGTGG Z GGTGGTTGTGGTGGTGGTGG	8949.17

Cy3-1642-145	Cy3-GGTGGTGGZGGTTGTGGTGGTGGTGG	8949.17
Cy3-1642-146	Cy3-GGTGGTGGTGGZTGTGGTGGTGGTGG	8949.17
Cy3-1642-147	Cy3-GGTGGTGGTGGTGTGGTGGTGGTGG	8949.17
Cy3-1642-148	Cy3-GGTGGTGGTGGTTGTGGTGGTGGTGG	8949.17
Cy3-1642-149	Cy3-GGTGGTGGTGGTTGTGGZGGTGGTGG	8949.17
Cy3-1642-150	Cy3-GGTGGTGGTGGTTGTGGTGGZGGTGG	8949.17
Cy3-1642-151	Cy3-GGTGGTGGTGGTTGTGGTGGTGGZGG	8949.17
Cy3-1642-152	Cy3-GGTGGTGGTGGTTGTGGTGGZGGZGG	9118.34
Cy3-1642-153	Cy3-GGTGGTGGTGGTTGTGGZGGTGGZGG	9118.34
Cy3-1642-154	Cy3-GGTGGTGGTGGTTGTGGTGGTGGZGG	9118.34
Cy3-1642-155	Cy3-GGTGGTGGTGGTGTGGTGGTGGZGG	9118.34
Cy3-1642-156	Cy3-GGTGGTGGTGGZTGTGGTGGTGGZGG	9118.34
Cy3-1642-157	Cy3-GGTGGTGGZGGTTGTGGTGGTGGZGG	9118.34
Cy3-1642-158	Cy3-GGTGGZGGTGGTTGTGGTGGTGGZGG	9118.34
Cy3-1642-159	Cy3-GGZGGTGGTGGTTGTGGTGGTGGZGG	9118.34
Cy3-1642-160	Cy3-GGTGGTGGTGGZZGTGGTGGTGGZGG	9287.51
Cy3-1642-161	Cy3-GGTGGTGGTGGZZGTGGTGGTGGTGG	9118.34
Cy3-1642-162	Cy3-GGTGGTGGTGGZZGZGGTGGTGGTGG	9287.51
Cy3-1642-163	Cy3-GGTGGTGGTGGZZGTGGZGGTGGTGG	9287.51
Cy3-1642-164	Cy3-GGTGGTGGTGGZZGTGGTGGZGGTGG	9287.51
Cy3-1642-165	Cy3-GGTGGTGGZGGZZGTGGTGGTGGTGG	9287.51
Cy3-1642-166	Cy3-GGTGGZGGTGGZZGTGGTGGTGGTGG	9287.51
Cy3-1642-167	Cy3-GGZGGTGGTGGZZGTGGTGGTGGTGG	9287.51
Cy3-1642-168	Cy3-GGTGGTGGZGGTTGTGGTGGTGGTGG	9118.34
Cy3-1642-169	Cy3-GGTGGZGGTGGTTGTGGZGGTGGTGG	9118.34
Cy3-1642-170	Cy3-GGZGGTGGTGGTTGTGGTGGZGGTGG	9118.34
Cy3-1642-171	Cy3-GGTGGZGGZGGTTGTGGZGGZGGTGG	9625.85
Cy3-1642-172	Cy3-GGZGGZGGZGGTTGTGGZGGTGGZGG	9795.02
Cy3-1642-173	Cy3-ZGGTGGTGGTGGTTGTGGTGGTGGTGGZ	9726.74

Cy3-1642-174	Cy3-CC Z CC Z CC Z CC Z Z C ZCC Z CC Z CC Z CC	9622.02
Cy3-1642-175	Cy3-CC Z CC Z CC Z CC T TCTCC Z CC Z CC Z CC	9114.51
Cy3-1642-176	Cy3-CCTCCTCCTCC Z Z C ZCCTCCTCCTCC	8607

Z = NapdU

(Table 5) A list of modified AS1411 having hydrophobic groups such as 4-PB at 5 position of dU (5-(*N*-4-pyrrolebenzylcarboxyamide)-2'-deoxyuridine, 4-PBdU)

Comp. No.	Cy3-labeled Modified dU-AS1411 sequence	MS
Cy3-1642-177	Cy3-GG Z GG Z GG Z GG Z Z G ZGG Z GG Z GG Z GG	10437.26
Cy3-1642-178	Cy3-GG Z GG Z GG Z GGTTGTGG Z GG Z GG Z GG	9884.84
Cy3-1642-179	Cy3-GG Z GG Z GGTGGTTGTGGTGG Z GG Z GG	9516.56
Cy3-1642-180	Cy3-GGTGG Z GG Z GGTTGTGG Z GG Z GGTGG	9516.56
Cy3-1642-181	Cy3-GG Z GGTGGTGGTTGTGGTGGTGG Z GG	9148.28
Cy3-1642-182	Cy3-GGTGG Z GGTGGTTGTGGTGG Z GGTGG	9148.28
Cy3-1642-183	Cy3-GGTGGTGG Z GGTTGTGG Z GGTGGTGG	9148.28
Cy3-1642-184	Cy3-GG Z GGTGG Z GGTTGTGG Z GGTGG Z GG	9516.56
Cy3-1642-185	Cy3-GGTGGTGGTGGT Z GTGGTGGTGGTGG	8964.14
Cy3-1642-186	Cy3-GGTGGTGGTGG Z Z G ZGGTGGTGGTGG	9332.42
Cy3-1642-187	Cy3-GGTGGTGGTGG Z TG Z GGTGGTGGTGG	9148.28
Cy3-1642-188	Cy3-GG Z GGTGGTGGTTGTGGTGGTGGTGG	8964.14
Cy3-1642-189	Cy3-GGTGG Z GGTGGTTGTGGTGGTGGTGG	8964.14
Cy3-1642-190	Cy3-GGTGGTGG Z GGTTGTGGTGGTGGTGG	8964.14
Cy3-1642-191	Cy3-GGTGGTGGTGG Z TGTGGTGGTGGTGG	8964.14
Cy3-1642-192	Cy3-GGTGGTGGTGGT Z GTGGTGGTGGTGG	8964.14
Cy3-1642-193	Cy3-GGTGGTGGTGGTTG Z GGTGGTGGTGG	8964.14
Cy3-1642-194	Cy3-GGTGGTGGTGGTTGTGG Z GGTGGTGG	8964.14
Cy3-1642-195	Cy3-GGTGGTGGTGGTTGTGGTGG Z GGTGG	8964.14
Cy3-1642-196	Cy3-GGTGGTGGTGGTTGTGGTGGTGG Z GG	8964.14

Cy3-1642-197	Cy3-GGTGGTGGTGGTTGTGGTGGZGGZGG	9148.28
Cy3-1642-198	Cy3-GGTGGTGGTGGTTGTGGZGGTGGZGG	9148.28
Cy3-1642-199	Cy3-GGTGGTGGTGGTTGZGGTGGTGGZGG	9148.28
Cy3-1642-200	Cy3-GGTGGTGGTGGTZGTGGTGGTGGZGG	9148.28
Cy3-1642-201	Cy3-GGTGGTGGTGGZTGTGGTGGTGGZGG	9148.28
Cy3-1642-202	Cy3- GGTGGTGGZGGTTGTGGTGGTGGZGG	9148.28
Cy3-1642-203	Cy3-GGTGGZGGTGGTTGTGGTGGTGGZGG	9148.28
Cy3-1642-204	Cy3-GGZGGTGGTGGTTGTGGTGGTGGZGG	9148.28
Cy3-1642-205	Cy3-GGTGGTGGTGGZZGTGGTGGTGGZGG	9332.42
Cy3-1642-206	Cy3-GGTGGTGGTGGZZGTGGTGGTGGTGG	9148.28
Cy3-1642-207	Cy3-GGTGGTGGTGGZZGZGGTGGTGGTGG	9332.42
Cy3-1642-208	Cy3-GGTGGTGGTGGZZGTGGZGGTGGTGG	9332.42
Cy3-1642-209	Cy3-GGTGGTGGTGGZZGTGGTGGZGGTGG	9332.42
Cy3-1642-210	Cy3-GGTGGTGGZGGZZGTGGTGGTGGTGG	9332.42
Cy3-1642-211	Cy3-GGTGGZGGTGGZZGTGGTGGTGGTGG	9332.42
Cy3-1642-212	Cy3-GGZGGTGGTGGZZGTGGTGGTGGTGG	9332.42
Cy3-1642-213	Cy3-GGTGGTGGZGGTTGZGGTGGTGGTGG	9148.28
Cy3-1642-214	Cy3-GGTGGZGGTGGTTGTGGZGGTGGTGG	9148.28
Cy3-1642-215	Cy3-GGZGGTGGTGGTTGTGGTGGZGGTGG	9148.28
Cy3-1642-216	Cy3-GGTGGZGGZGGTTGZGGZGGZGGTGG	9700.7
Cy3-1642-217	Cy3-GGZGGZGGZGGTTGZGGZGGTGGZGG	9884.84
Cy3-1642-218	Cy3-ZGGTGGTGGTGGTTGTGGTGGTGGZ	9756.68
Cy3-1642-219	Cy3-CCZCCZCCZCCZCCZCCZCCZCC	9756.75
Cy3-1642-220	Cy3-CCZCCZCCZCCTTCTCCZCCZCCZCC	9204.33
Cy3-1642-221	Cy3-CCTCCTCCTCCZCZCCTCCTCCTCC	8651.91

Z = 4-PBdU

1.2: Synthesis of Cy3-labeled modified dU AS1411 and GRO29A

Cy3-labeled AS1411(GGTGGTGGTGGTTGTGGTGGTGGTGG, SEQ ID NO:

2) and GRO29A(TTTGGTGGTGGTGGTTGTGGTGGTGGTGG, SEQ ID NO: 1), and Cy3-labeled modified dU-containing AS1411 and GRO29A were synthesized using a Mermade 12 DNA synthesizer (BioAutomation Manufacturing, Irving, TX,) with standard solid phase phosphoramidite chemistry. 5-(*N*-benzylcarboxyamide)-2'-deoxyuridine (BzdU), 5-(*N*-naphthylcarboxyamide)-2'-deoxyuridine (NapdU), and 5-(*N*-4-Pyrrolebenzylcarboxyamide)-2'-deoxyuridine(4-PBdU)-phosphoramidite were offered by Samchully Pharmaceutical (Seoul, Korea). All oligonucleotide syntheses were performed in house.

All oligonucleotides were synthesized on functionalized controlled pore glass (CPG) synthesized using a Mermade 12 DNA synthesizer (BioAutomation Manufacturing, Irving, TX) with 0.067 M solution of the modified dU(BzdU, NapdU or 4-PBdU)-amidite in anhydrous acetonitrile. For incorporation of dA, dG, dC and dT residues standard phosphoramidites with exocyclic amino groups protected with benzoyl group (for dA and dC) and isobutyryl group (for G) were used. For incorporation of modified dU-amidite, phosphoramidite solution was delivered in two portions, each followed by a 5 min coupling wait time. Oxidation of the internucleotide phosphate to phosphate was carried out using an oxidizer [tetrahydrofuran (THF), pyridine, 0.02 M iodine and water] with waiting time. All other steps in the protocol supplied by the manufacturer were used without modification. The coupling efficiencies were >97%. After completion of the synthesis, the next step is treatment with the cleavage solution(*t*-butylamine:methanol:water, 1:1:2) at 70°C for 5 hours to hydrolyze the ester linking the DNA to the support and to remove protecting groups from the purine and pyrimidine bases and followed by freezing, filtration, and speed-vac evaporation to dryness.

Crude oligonucleotides were purified by high performance liquid chromatography (AKTA basic HPLC, XBridge OST C18 10x50mm, A = 100 mM buffer triethylammoniumbicarbonate (TEAB), pH = 7, B = acetonitrile, 8% to 40% B in 20 min, flow 5 mL min⁻¹, at 65°C, λ = 254 and 290nm). Purified aptamers were precipitated by ethanol and desalted by Centricon (Millipore Bedford, MA). Finally, desalted aptamers were resuspended in water or phosphate buffered saline and sterilized

by filtration through a 0.2- μ m syringe filter. Molecular weight and purity of each aptamer was checked by Q-TRAP 2000 ESI-MS spectroscopy (Applied Biosystems foster city, CA) and P/ACE™ 2000 capillary gel electrophoresis(Beckman coulter. fullerton, CA).

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Example 2: Affinity of the aptamer to nucleolin

2.1: Cell culture

C6 cells (American type culture collection), which are a rat glioma cell lines, were maintained in DMEM (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Grand Island, NY), 10 U/ml penicillin (Invitrogen, Grand Island, NY), and 10 μ g/ml streptomycin in a 5% CO₂-humidified chamber at 37°C. The cells were cultured in multiwell chamber slides overnight or 2 days till they reach about 50–80% confluence. After confluent of cells 90-100%, cells were aspirated off media with transfer pipettes and washed with PBS (1× Phosphate Buffered Saline) briefly. After trypsinization, cells were collected by standard culture media and 1/5 cells that were centrifuged 1000 rpm for 5 min were transferred into T75 flask holding 10 ml media.

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2.2: Protein assay

To normalize fluorescence signals of forty-seven Cy3-labeled BzdU-containing GRO29A and eighteen Cy3-labeled NapdU-containing GRO29A compounds, cells treated with each compound were collected with 120 μ l PBS buffer after trypsinization and followed with BCA protein assay (Thermo Fisher Scientific Inc. Waltham, MA). Then, the collected cells were moved into 96-microplate well and treated mixture of reagent A and B (1:50 (v/v)) and incubated at 37°C for 30 min. After the buret reaction, the absorbance at or near 562 nm reader measured on a plate.

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2.3: Fluorescence intensity

To determine the targeting efficiency of the cancers, fluorescence intensities of forty-seven Cy3-labeled BzdU-containing GRO29A and eighteen Cy3-labeled NapdU-

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containing GRO29A compounds were quantified to evaluate their targeting efficiency at C6 cells by the Varioskan Flash spectral scanning multimode reader (Thermo Fisher Scientific Inc. Waltham, MA; excitation: 535 nm, scanning wavelength: 570 nm with a band width: 12 nm). C6 cells were seeded 1×10^5 cell density onto Magne to
5 FACTION 24 plate (Chemicell, GmbH, Germany) and caring at a 5% CO₂-humidified chamber. After 24 hours of grown, these seeded cells were incubated in DMEM (Dubelocos' modified essential media) with 20 nM of Cy3-labeled GRO29A or AS1411, or 20 nM of each of the Cy3-labeled modified dU-containing GRO29A or AS1411 compounds at for 30 minute at 4 °C for decreasing non-specific binding during 30 min
10 and rinsed by PBS (1×), then replaced to 200 μ l Tris buffer, and then treated with each of 47 different compounds (20 pmole). Then, seeded cells was washed with PBS (phosphate buffered saline) two times each for 10 min at RT using shaking incubation (30 rpm) to remove the unbound Cy3-labeled modified dU-containing GRO29A or AS1411 compounds, and subjected to trypsinization to detach from the plate surface.
15 These cells were collected by PBS (1×) (120 μ l) and transferred into 96-well plate (Chemicell, GmbH, Germany) for measurement of fluorescence intensity (100 μ l).

The fluorescence intensities of the Cy3-labeled modified dU-containing GRO29A or AS1411 compounds, targeting the nucleolin proteins expressed in the cellular membrane of the C6 cells, were quantified and normalized by units of the cells
20 measured by the Bradford protein assay using Varioskan Flash spectral scanning multimode reader.

2.4: Confocal laser microscopy assay

To further validate the increased binding affinity of modified dU-containing
25 GRO29A or AS1411 by confocal microscopy analysis. Confocal microscopy imaging a laser scanning microscope (Carl Zeiss, Inc., Weimer, Germany; HFT 405/488 nm, DAPI imaging: 420-480 nm, Cy3-labeled compounds: 488/543) was used and each C6 cell was seeded 1×10^5 cells onto 12 mm sterile coverslip in 24-well plate. After 24 hr, C6 cells were incubated in PBS for 30 min at 4 °C with Cy3- GRO29A or AS1411, or
30 Cy3-(5-BzdU)-modified GRO29A or AS1411 compounds (respectively, 20nM).

To remove the unbound conjugates, the cells were washed three times during 10 min using shaking incubation (30 rpm) in PBS (1×) and fixed with 200ul of 3.7 % formaldehyde solution (Sigma, Saint Louis, MO) that was treated 200 μ l into cells and incubated at shaking incubation (20 rpm) each for 20 min. After washed three times
5 with PBS for 10 min into shaking incubation, this was followed by staining of the nucleus with a 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; emission: 460 nm, blue color) using the mounting solution (Vector Laboratories, Inc., Burlingame, CA). Fluorescent imaging of targeting C6 cells was visualized by red color. All fluorescence data were acquired at an excitation of 535 nm and emission of 570 nm.
10 The side of fixed cells onto coverslip was put on 10 μ l of mounting solution (Vector Resources Inc, Torrance, CA. USA). The confocal images were acquired at low magnifications (200×).

2.5: Statistical analysis

15 Fold ratio of fluorescence activity for 47 different compounds of Cy3-labeled BzdU-containing GRO29A and eighteen Cy3-labeled NapdU-containing GRO29A and the Cy3-labeled CRO29A was normalized to the fluorescence signals of Cy3-AS1411 and p-values were calculated using the Student's *t*-test.

20 2.6: Results

The results of fluorescence analysis for several Cy3-labeled BzdU-containing GRO29A compounds are shown in Fig. 2. Fig. 2 shows the results of fluorescence analysis of Cy3-labeled BzdU-containing GRO29A compounds targeting C6 cells. The fluorescence of the Cy3-labeled BzdU-containing GRO29A compounds that were
25 bound and targeted the nucleolin protein in the C6 cells was quantified. The X-axis indicates numbers of compounds. These data are presented as the means $\pm$ SD calculated from quadruple wells. All fluorescence data were obtained at an excitation of 488 nm and emission of 543 nm. Fig. 2 presents comparison of the fluorescent intensity of the Cy3-labeled GRO29A with excitation at 488 nm and emission at
30 543 nm, showing that Cy3-labeled BzdU-containing GRO29A compounds showed

either a slightly or a significantly greater fluorescent activity in the C6 cells compared to Cy3-labeled GRO29A or CRO29A.

The fold ratio of which the fluorescence signals for 47 different compounds of Cy3-labeled BzdU-containing GRO29A was normalized to that of Cy3-labeled
5 GRO29A showed that seven different compounds, Compound No. 1642-11, 1642-13, 1642-19, 1642-30, 1642-39, 1642-49, and 1642-51 had about a 1.5 fold or more binding affinity to C6 cells as shown in Fig. 2. In particular, No. 1642-19 compound had approximately 2.5 fold higher fluorescent signals than Cy3-labeled GRO29A. The statistical analysis using Student-t test demonstrated that p-value with higher than 0.05
10 was found in 18 different compounds of Cy3-labeled BzdU-containing GRO29A including No. 1642-19, 1642-39, 1642-49, and 1642-51. This result implied that BzdU modification, such as compounds No. 1642-19, 1642-39, 1642-49, and 1642-51, significantly increased targeting affinity to C6 cells.

To further validate the increased binding affinity of Cy3-labeled BzdU-
15 containing GRO29A by confocal microscopy analysis, compounds No. 1642-19, 1642-39 and 1642-51 were incubated and visualized in C6 cells with Cy3-labeled GRO29A and Cy3-labeled CRO29A (negative control). Twenty 20 nM of each compound was targeted in the C6 cells. The results of confocal microscopy analysis for compounds No. 1642-19, 1642-39, and 1642-51 were shown in Fig. 3. As shown in Fig. 3, comparison
20 of the phase-contrast image and the nuclear DAPI staining revealed that numbers 1642-19, 1642-39, and 1642-51, and Cy3-labeled GRO29A were extensively bound to the plasma membrane of the C6 cells while the Cy3-labeled CRO29A was not clearly visualized in the C6 cells. The targeting affinity to C6 cells, shown in Fig. 3, demonstrated that compounds 1642-19, 1642-39, and 1642-51 had better targeting
25 affinity than did Cy3-labeled GRO29A. The 1642-19 compound showed the highest fluorescent brightness in C6 cells.

To test functional activity of BzdU-containing GRO29A, the compounds numbers 1642-39, 1642-51 and 1642-19, in other cancer cells, the inventors first extended cancer targeting assay by selecting another cancer cells, HeLa (human cervix
30 cancer cell line, ATCC), and a normal healthy cell line, CHO (chinese hamster ovary

cell line, ATCC). The measurement of fluorescence intensity was performed as described above for C6 cells. The obtained results are shown in Fig. 4.

Fig. 4 shows the results of fluorescence analysis of numbers 1642-39, 1642-51, 1642-19 and Cy3-labeled GRO29A, and Cy3-labeled CRO29A (the control form of GRO29A labeled with Cy3). Quantitative fluorescence intensity in HeLa and CHO cells. Data are represented as means $\pm$ standard error of means (* p <.05,** P <.005 unpaired t -test). Similar results with C6 cell quantitative fluorescence intensity showed that compounds 1642-39, 1642-51 and 1642-19 had the higher binding affinity for the HeLa cells than the Cy3-labeled GRO29A, while the CRO29A had no significant fluorescent signal in HeLa cells. Especially, the 1642-19 compound had approximately a 2.3-fold higher fluorescent activity in HeLa cells than the Cy3-labeled GRO29A. However, in CHO cells, the compounds including numbers 1642-39, 1642-51 and 1642-19, Cy3-labeled CRO29A, and Cy3-labeled GRO29A showed undetectable fluorescence intensity. These results indicate that BzdU-containing GRO29A high-specifically targets to cancer cell compared with GRO29A.

As shown in Fig. 4, there was no significant difference in binding affinity to CHO cells compared with the mutant. To confirm that the modified GRO29A has increased affinity compared to non-modified GRO29A other cells than C6, confocal microscopy analyses for HeLa cells and CHO cells were performed as the same method described above. The observed confocal microscopy images in HeLa cells and CHO cells are shown in Fig. 5A (Hela cell) and 5B (CHO cell). Confocal microscopy analysis validated that the compounds numbers 1642-39, 1642-51 and 1642-19 had extensively and better binding affinity to the plasma membrane of the HeLa cells than the Cy3-labeled GRO29A, while the CRO29A was not significantly visualized in the HeLa cells. There was no significant difference in binding affinity to CHO cells compared with the mutant. Confocal microscopy analysis validated that the compounds numbers 1642-39, 1642-51 and 1642-19 had extensively and better binding affinity to the plasma membrane of the HeLa cells than the Cy3-labeled GRO29A, while the CRO29A was not significantly visualized in the HeLa cells. As expected, the compounds including numbers 1642-39, 1642-51 and 1642-19, CRO29A, and GRO29A

were not clearly visualized in CHO cells.

The results for Cy3-labeled NapdU-containing GRO29A compounds are shown in Fig. 7. Fig. 7 shows the results (fluorescence intensity) measured by fluorescence analysis of Cy3-labeled NapdU-containing GRO29A compounds targeting C6 cells with
5 excitation 488 nm and emission 543nm 4 different compounds. The fluorescence quantification of 18 different Cy3-labeled NapdU-containing GRO29A compounds which were bound and target nucleolin protein in C6 cells. X-axis indicated No. of compounds. These data are presented as means $\pm$ SD calculated from quadruple wells. As shown in Fig. 7, compound nos. 1642-70, 1642-71, 1642-72 and 1642-73 had
10 extensively and better binding affinity to the plasma membrane of the C6 cells than the Cy3-labeled GRO29A.

Example 3: FACS (fluorescence activated cell sorter) analysis

3.1: Cell culture

15 All cultures were grown in a humidified incubator maintained at 37°C with 95% air/5% CO₂. C6 (Glioma cancer), MDA-MB231 (Breast cancer), MG63 (osteosarcoma), U87MG (Glioma cancer), OVCAR-3 (Ovarian carcinoma), and HeLa (Cervical Carcinoma) human cancerous cells were obtained from the American Type Culture Collection (ATCC) and were propagated in DMEM medium supplemented with
20 10% fetal bovine serum (FBS), penicillin (100IUmmL⁻¹), and streptomycin (100IUmmL⁻¹). AGS (Gastric cancer, ATCC) and HepG2 (Liver cancer, ATCC) cells were grown in RPMI1640 and MEM (Invitrogen, Carlsbad, CA), respectively. NIH3T3 cells (Normal, ATCC) were cultured in DMEM supplemented with 10% FBS and antibiotics (100IUmmL⁻¹ penicillin, 100IUmmL⁻¹ streptomycin, Invitrogen,
25 Carlsbad, CA). The modified aptamer was dissolved in culture media before addition to the cell cultures for the cell proliferation assay.

3.2: FACS (fluorescence activated cell sorter) analysis

Cells monolayers were detached by 2mM EDTA, filtered with 40 μ m Cell
30 strainer (BD Falcon), and then washed with HBSS solution (Gibco). The each Cy3-

labeled aptamer (AS1411 or modified dU containing AS1411, 100pmol) was incubated with cells respectively in 200 μ L of HBSS solution on ice for 60 min. Cells were washed three times with 500 μ L of HBSS solution and suspended in 1mL of 1% paraformaldehyde solution. The fluorescence was determined with FACSCalibur (BD Biosciences) by counting 10,000 events.

The obtained results in various cell lines are shown in Figs 8-21. As shown in Figs 8-21, the intensities corresponding to the peaks for the modified dU-containing aptamer (modified dU containing AS1411) is higher than those of non-modified aptamer (AS1411), indicating that the modified aptamer has a higher specificity to cancer cells compared to the non-modified aptamer.

Figs. 22-26 shows the results of quantification of the results of FACS analysis for various cell lines as shown in Figs 8-21, indicating that chemical modification of thymidine at the particular region of AS1411 with Bz, Nap, or 4-PB, would form more stable G-quadruplex structure via hydrophobic cavities and enhance the potential binding affinity of AS1411 to cancer cells. As shown in Fig. 22-26, A1 modified dU-containing AS1411 (GGZGGZGGZGGZZGZGGZGGZGGZGG), a central double modified dU-containing AS1411 (GGTGGTGGTGGZZGTGGTGGTGGTGG) and several central double and a more modified dU-containing AS1411 (GGTGGTGGTGGZZGTGGTGGTGGZGG, GGTGGTGGTGGZZGZGGTGGTGGTGG, GGTGGTGGZGGZZGTGGTGGTGGTGG and GGZGGTGGTGGZZGTGGTGGTGGTGG) increase binding to nucleolin on various cancer cell lines compare to AS1411.

In particular, both of central double modified dU-containing and a more modified dU-containing AS1411 have been shown same or similar binding to various cancer cell lines. The activity between central double modification and a more modification of AS1411 were measured through the FACS analysis as described above, and the obtained results are shown in Fig. 23. A more modification on any position of central double modified dU-containing AS1411 was not increased binding to nucleolin on various cancer cell lines, indicating that the modification of the central region (2

bases) may be critical region for the modification of the aptamer to effect on the affinity of the aptamer to nucleolin.

Example 4: MR Imaging

5 C6 rat glioma cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (Invitrogen), supplemented with 10% heat-inactivated (65 °C for 20 min) fetal bovine serum (Invitrogen) with 1% antibiotics (Invitrogen), in a standard incubator (5% CO₂ atmosphere at 37 °C). 5×10⁶ cells of the cultured C6 cells were transplanted into subcutaneous tissue of both thigh of nude mice (male, BALB/c, 7-weeks old,
10 Chalsriver).

T2 axial images were obtained using 1.5-T MR imager (GE Medical Systems, Milwaukee, WI, USA) in animal coil box. During the experimentation, the tumor-bearing nude mice were intraperitoneal injections of 50 mL of a ketamine and xylazine (2:1) solution for anesthesia. The temperature and respirations of the tumor-bearing
15 nude mice were monitored by a rectal thermistor. The sequence parameters for repetitive time (TR) and echo time (TE) were 1400 and 55.8 ms, respectively.

MNP@SiO₂(RITC)-(PEG)/COOH/pro-N/NH₂ nanoparticles (MF, 2 mg/mL) were purchased from Biterials (Seoul, Korea) and prepared as previously described (17). Carboxyl moieties (1.1×10⁴/nanoparticle) of the MF particles (size; 50nm;
20 hydrodynamic diameter; 58.1 nm) were covalently linked to a 5'-NH₂-modified AS1411 aptamer (SEQ ID NO: 2) or the modified dU containing AS1411 aptamer using N-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC) (MF:aptamer molar ratio in conjugation reaction, 1:3, Sigma) for 1 h at room temperature. The AS1411-MF
25 conjugates were washed off by centrifugation at 22,250g for 10 min and resuspended in selection buffer solution (50 mM Tris-HCl, pH 7.4). Amine groups (6.4×10⁴/nanoparticle) protected by the Fmoc group were released by 20% piperidine (Sigma) in an N,N-dimethylformamide solution (Sigma). After 1 h of incubation, the AS1411-MF particles were washed off twice with Tris buffer (pH 7.4) and briefly sonicated.

30 The AS1411-MF particle and modified dU containing AS1411(central double

NapdU-containing AS1411(1642-161))-MF particle were suspended in PBS, and injected into the tumor-bearing nude mice through tail-vein injection in the amount of 5mg/kg of body weight. T2-weighted MR images were obtained from the both thigh of the tumor-bearing nude mice before and 24 hr after intravenous injection of AS1411-MF or AS1411(1642-161)-MF, and shown in Fig. 27. As shown in Fig. 27, T2-weighted MR images from tumor-bearing mice injected with modified AS1411-MF showed the AS1411-MF particles as bigger block spots than that of AS1411. No T2-negative images were observed in the control AS1411-MF particle-injected tumor-bearing mice.

Example 5: Cell proliferation assay

To test another functional activity of the chemically modified nucleolin aptamer on tumor cell death, cell proliferation test was performed by MTT assay, based on the fact that nucleolin aptamer has antiproliferative effects by specifically binding to the nucleolin transmembrane protein in cancer cells.

To determine cell survival after exposure to the chemically modified aptamer for 5 days, measurement of cell proliferation was performed colorimetrically by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) assay, using the CellTiter96 Aqueous One Solution Reagent (Promega). Cells were seeded onto 96-well plates at 4×10^5 cells well⁻¹ in 100 μ L of medium, and the tested modified aptamer was added to allow to attach for 24 hr. The cell monolayer was washed with phosphate buffered saline (PBS) to remove unattached cells, and the cells were maintained in serum-free medium (SFM) for 24h, and then washed with PBS. Fresh SFM with the modified aptamer was added, and the cells were incubated for an additional 5 days. Subsequently, the cells were exposed to MTS for 15 min and absorbance was measured using a microplate reader (Dynex Technology, Chantilly, VA, USA) at an optical density (OD) of 490 nm. OD values from the control cells were designated 100% as a standard.

For test the activity CRO29A on cell proliferation, 4M of each compound, numbers 1642-39, 1642-51 and 1642-19, Cy3-labeled CRO29A, and Cy3-labeled

GRO29A were added directly to MDA-MB231 cells (Breast cancer, ATCC) and incubated for 1 day. The cell viability(%) was measured based on that of control (100%). The control is a cell group without treatment of aptamer, and the results are shown in Fig. 6.

5 Fig. 6 shows anti-proliferation effects measured by MTT assay. 4mM of each compounds was treated at 2×10^5 C6 cells per well. Data are represented as means $\pm$ standard error of means (**P < .005 unpaired t-test). The compounds numbers 1642-51 and 1642-19 showed significantly higher antiproliferative effect than the Cy3-labeled GRO29A, representing 55% and 65% of cell viability.

10 To confirm that the central double modification of the aptamer is also critical in treating cancer, the effect of central double NapdU-containing AS1411 (4 μ M) on the viability of MDA-MB231 breast cancer cells (ATCC) were measured by the method as described above, and the results are shown in Fig. 28 (wherein, '6' refers to NapdU). The results indicate that central double NapdU-containing AS1411 showed higher
15 inhibition effect than AS1411.

 The inventors synthesized various compounds of Cy3-labeled modified dU-containing AS1411(or GRO29A) with single or multiple modifieddUs to increase their binding affinity to nucleolin proteins in the cell membranes of cancer cells. The
20 quantification of fluorescent signals demonstrated that a variety of chemically modified AS1411 compounds using modifieddU had varied binding affinity to cancer cells. The number and position of substituents in the AS1411(or GRO29A) nucleotides were compared with the original sequences of AS1411(or GRO29A). Our statistical analysis and confocal microscopy imaging showed that at least three compounds, numbers 1642-
25 19 (TTTGGTGGTGGTGGTTGTGGTGGTGGZGG, Z = BzdU), 1642-39 (TTTGGTGGTGGTGGZZGTGGTGGTGGTGG, Z = BzdU), and 1642-51 (ZTZZGGTGGTGGTGGZZGTGGTGGTGGTGG, Z = BzdU) out of 47 different Cy3-labeled BzdU-containing GRO29A, resulted in a significant increase in targeting the C6 cells. To assess whether the number and position of the BzdU incorporated into
30 GRO29A had the influence of targeting and binding the C6 cells, the chemically

modified sequences of the 47 different compounds of Cy3-labeled BzdU-containing GRO29A were compared with regard to their fluorescent activity in targeting of the C6 cells. For incorporation of NapdU into GRO29A, some central double modification, No. 1642-70(ZTTGGTGGTGGTGGZZGTGGTGGTGGTGG), 1642-71
 5 TZZGGTGGTGGTGGZZGTGGTGGTGGTGG), 1642-72(ZTZGGTGGTGGTGGZZGTGGTGGTGGTGG) and 1642-73(ZZGGTGGTGGTGGZZGTGGTGGTGGTGG) had extensively and better binding affinity to the plasma membrane of the C6 cells than the Cy3-labeled GRO29A. There are 12 thymidine nucleotides in the GRO29A sequence that can be substituted with
 10 modifieddU such as BzdU and NapdU. One fixed incorporation of BzdU at the 12th thymidine, resulted in the highest binding affinity to the cancer cells and showed increased targeting affinity. In addition, most of the double incorporated BzdU or NapdU at the 7th and 8th thymidine, in the GRO29A compound, produced either a slight improvement or a significant improvement in the binding affinity for the C6 cells.
 15 Other random heavy modification of AS1411 did not result in a significant increase in the binding affinity for the C6 cells. These findings imply that chemical modification of thymidines at the central double region of GRO29A with modifieddU such as BzdU or NapdU forms a more stable G-quadruplex structure via hydrophobic cavities and enhances the potential binding affinity of GRO29A for cancer cells. At the results of
 20 FACS analysis with modifieddU-containing AS1411, central double modifieddU-containing AS1411(GGTGGTGGTGGZZGTGGTGGTGGTGG, Z = BzdU, NapdU and 4-PBdU) had extensively and better binding affinity to the nucleolin of cancer cell lines than AS1411. (see Fig. 29).

FIG. 29 shows a relation between structure and activity of central double
 25 modifieddU-containing AS1411 (or GRO29A). The position and structure of chemical modification in central double modification and the original sequence of AS1411 were drawn in G-quadruplex structure that normally forms by dimerization of AS1411 aptamers to bind to nucleolin protein.

30 The results of the examples highlight the fact that chemical modifications can

directly applied to alter existing aptamers thereby increasing their binding affinity for targets without a significant increase in time or labor for the SELEX procedure. Such chemically modified aptamers could be used as a valuable clinical tool for identifying serious cancer disease, in a very early stage, and evaluation of cancer therapy. However,
5 further analysis including the study of diverse existing aptamers and their targets as well as study of resistance to enzymatic degradation, biostability in vivo, and optimization of the number and positioning of the modifieddU such as BzdU, NapdU and 4-PBdU compounds in the sequence of the existing aptamers must be studied before in vivo application is considered for the detection and treatment of cancers.

10

WHAT IS CLAIMED IS:

1. Nucleolin-specific aptamer, which has the sequence of SEQ ID NO: 3, and one or more thymidines(T) present in the nucleotide sequence are independently substituted with a modified pyrimidine nucleoside, and wherein the modified pyrimidine nucleoside is deoxyuridine(dU), deoxycytidine(dC), uridine(U), or cytidine(C) having a hydrophobic group at 5' position:

NGGTGGTGGTGGTTGTGGTGGTGGN (SEQ ID NO: 3)

wherein each N is absent or 1 to 20 nucleosides, which is independently selected from the group consisting of adenosine(A), thymidine(T)/uridine(U), cytidine(C), and guanosine(G).

2. The nucleolin-specific aptamer according to Claim 1, which has the sequence of SEQ ID NO: 1 or 2, and one or more thymidines(T) present in the nucleotide sequence are independently substituted with a modified pyrimidine nucleoside, and wherein the modified pyrimidine nucleoside is deoxyuridine(dU), deoxycytidine(dC), uridine(U), or cytidine(C) having a hydrophobic group at 5' position.

3. The nucleolin-specific aptamer according to Claim 1, wherein the hydrophobic group is selected from the group consisting of a benzyl group, a naphthyl group, or a pyrrolebenzyl group.

4. The nucleolin-specific aptamer according to Claim 1, wherein the modified pyrimidine nucleoside is selected from the group consisting of 5-(N-benzylcarboxyamide)-2'-deoxyuridine(called BzdU), 5-(N-naphthylcarboxyamide)-2'-deoxyuridine(called NapdU), 5-(N-4-pyrrolebenzylcarboxyamide)-2'-deoxyuridine(called 4-PBdU), 5-(N-benzylcarboxyamide)-2'-deoxycytidine (called BzdC), 5-(N-naphthylcarboxyamide)-2'-deoxycytidine (called NapdC), 5-(N-4-pyrrolebenzylcarboxyamide)-2'-deoxycytidine (called 4-PBdC), 5-(N-benzylcarboxyamide)-2'-uridine(called BzU), 5-(N-naphthylcarboxyamide)-2'-

uridine(called NapU), 5-(*N*-4-pyrrolebenzylcarboxamide)-2'-uridine(called 4-PBU), 5-(*N*-benzylcarboxamide)-2'-cytidine (called BzC), 5-(*N*-naphthylcarboxamide)-2'-cytidine (called NapC), and 5-(*N*-4-pyrrolebenzylcarboxamide)-2'-cytidine (called 4-PBC).

5

5. The nucleolin-specific aptamer according to Claim 1, wherein at least two thymidines present in 9th to 18th positions of SEQ ID NO: 3 are substituted with the modified pyrimidine nucleoside, and the positions positions are counted starting from 'G' after 'N' at 5'-end.

10

6. The nucleolin-specific aptamer according to Claim 2, wherein at least two thymidines present in 12th to 18th positions of SEQ ID NO: 1 or 9th to 18th positions of SEQ ID NO: 2 are substituted with the modified pyrimidine nucleoside.

15

7. A composition for diagnosing a cancer, containing the nucleolin-specific aptamer of any one of Claim 1 to Claim 6.

20

8. The composition according to Claim 7, wherein nucleolin-specific aptamer is labeled by one or more selected from the group consisting of a fluorescence material, infrared material, quantum dots, ion oxide bead, PET probe, T1 MR probe, and T2 MR probe.

25

9. The composition according to Claim 7, wherein the cancer is selected from the group consisting of leukemias, lymphomas, myeloproliferative disorders, carcinomas of solid tissue, sarcomas, melanomas, adenomas, hypoxic tumors, squamous cell carcinomas of the mouth, throat, larynx, or lung, genitourinary cancers, hematopoietic cancers, head and neck cancers, and nervous system cancers, and benign lesions.

30

10. A composition for treating a cancer, containing the nucleolin-specific

aptamer of any one of Claim 1 to Claim 6 as an active ingredient.

11. The composition according to Claim 10, wherein the cancer is selected from the group consisting of leukemias, lymphomas, myeloproliferative disorders, carcinomas of solid tissue, sarcomas, melanomas, adenomas, hypoxic tumors, squamous
5 cell carcinomas of the mouth, throat, larynx, or lung, genitourinary cancers, hematopoietic cancers, head and neck cancers, and nervous system cancers, and benign lesions.

10 12. A composition for inhibiting nucleolin, containing the nucleolin-specific aptamer of any one of Claim 1 to Claim 6 as an active ingredient.

13. A composition for inhibiting hyperproliferation of cell caused by nucleolin, containing the nucleolin-specific aptamer of any one of Claim 1 to Claim 6 as
15 an active ingredient.

14. A pharmaceutical composition containing the nucleolin-specific aptamer of any one of Claim 1 to Claim 6.

Fig. 1

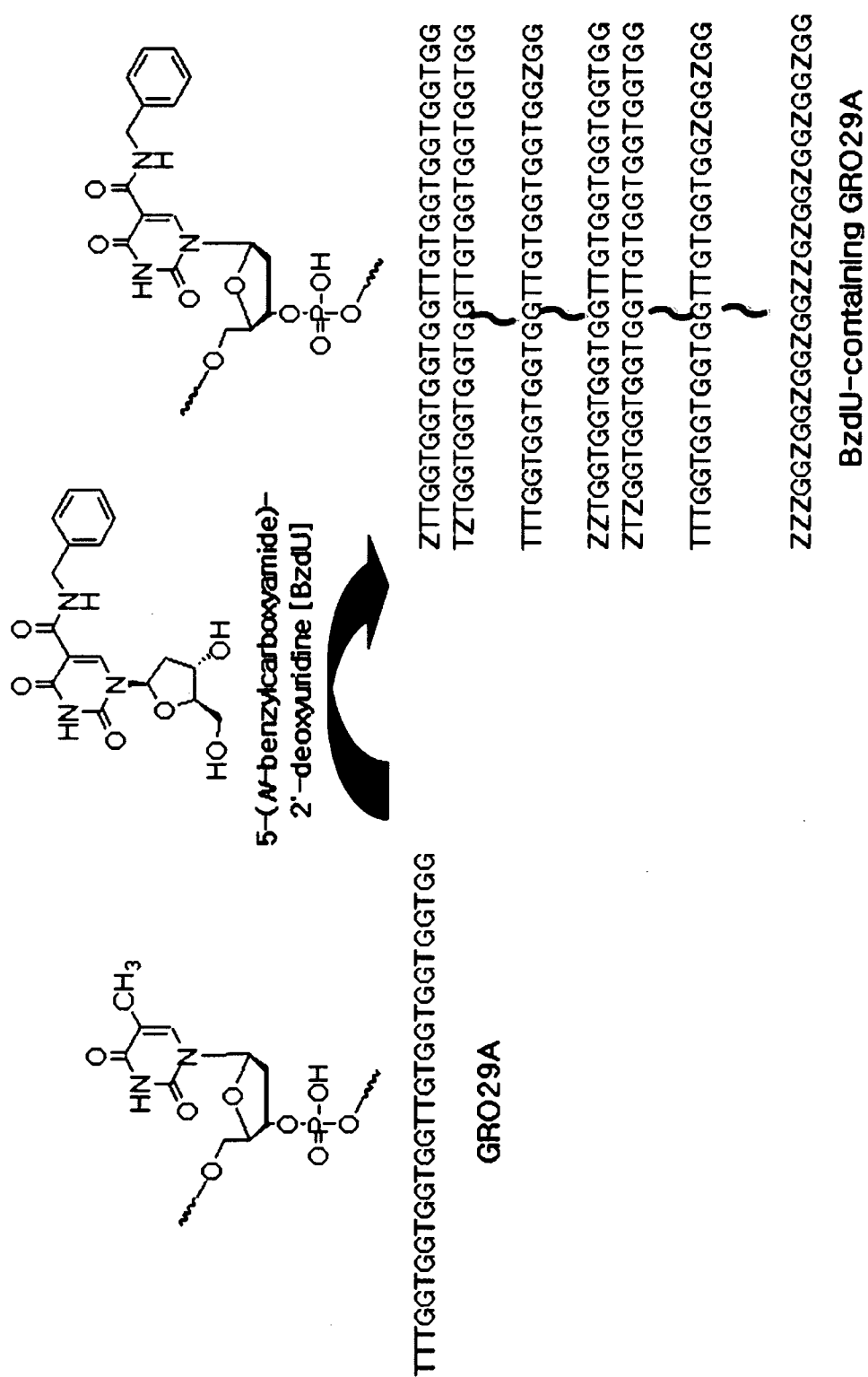


Fig. 2

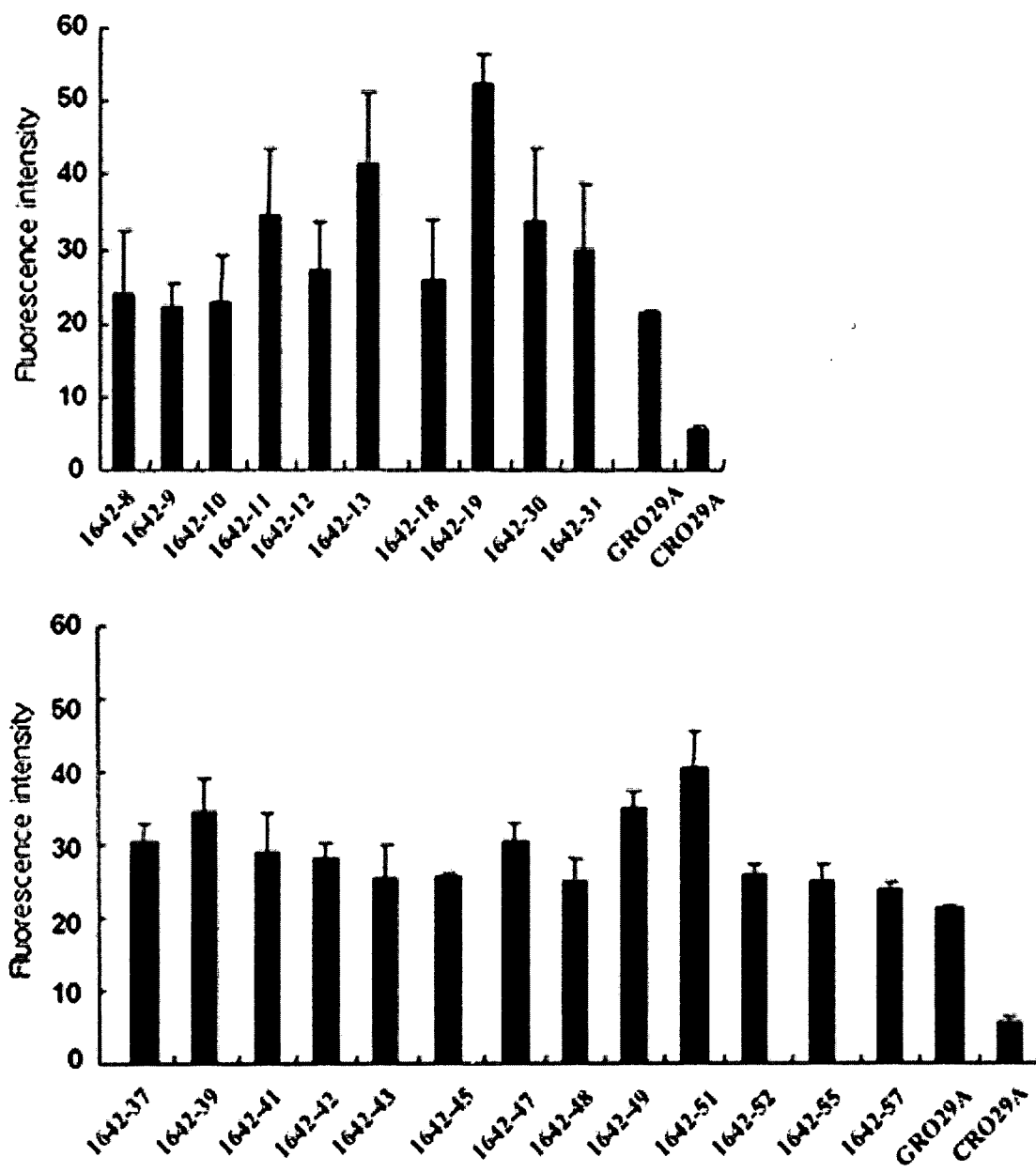


Fig. 3

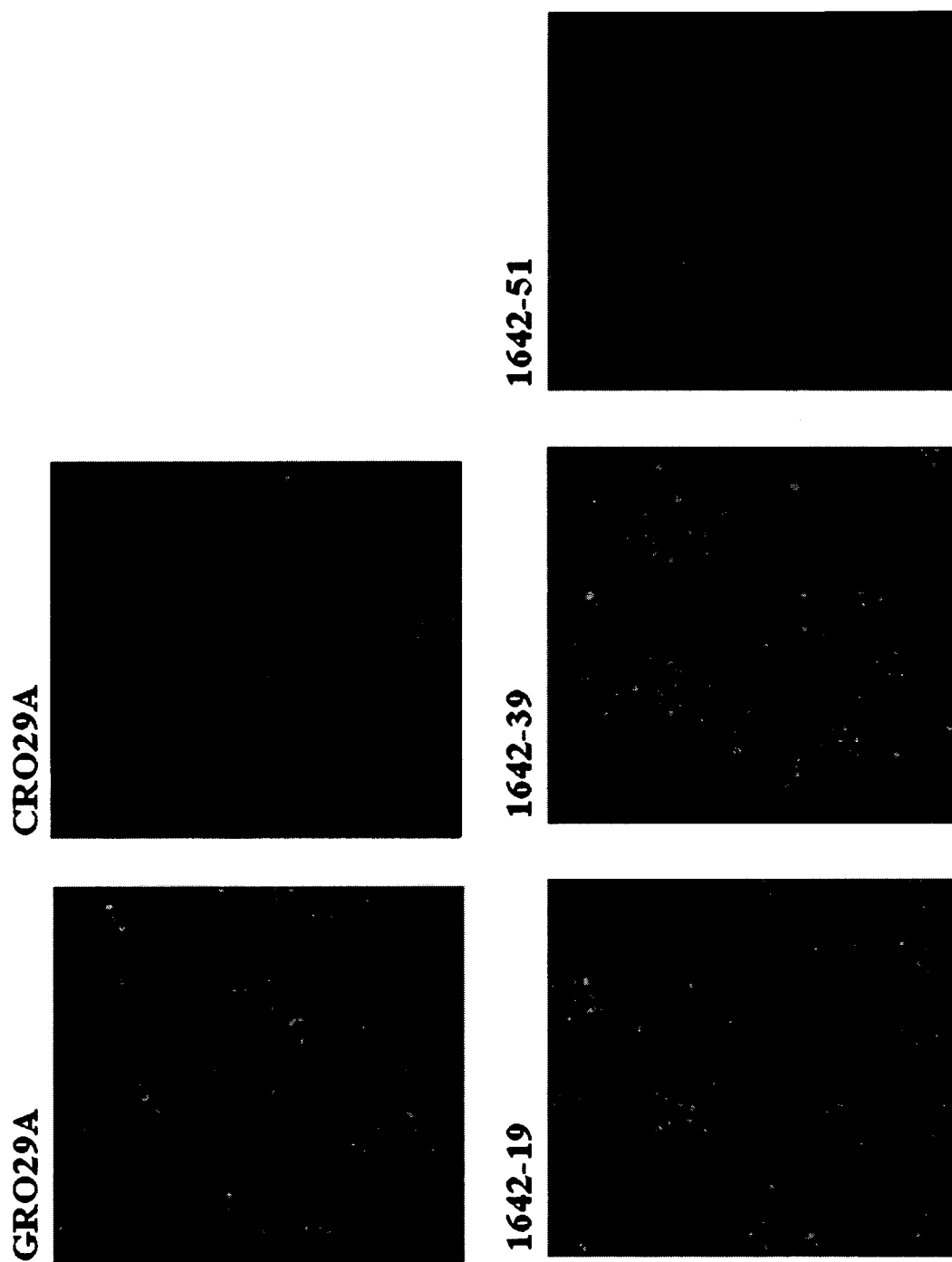


Fig. 4

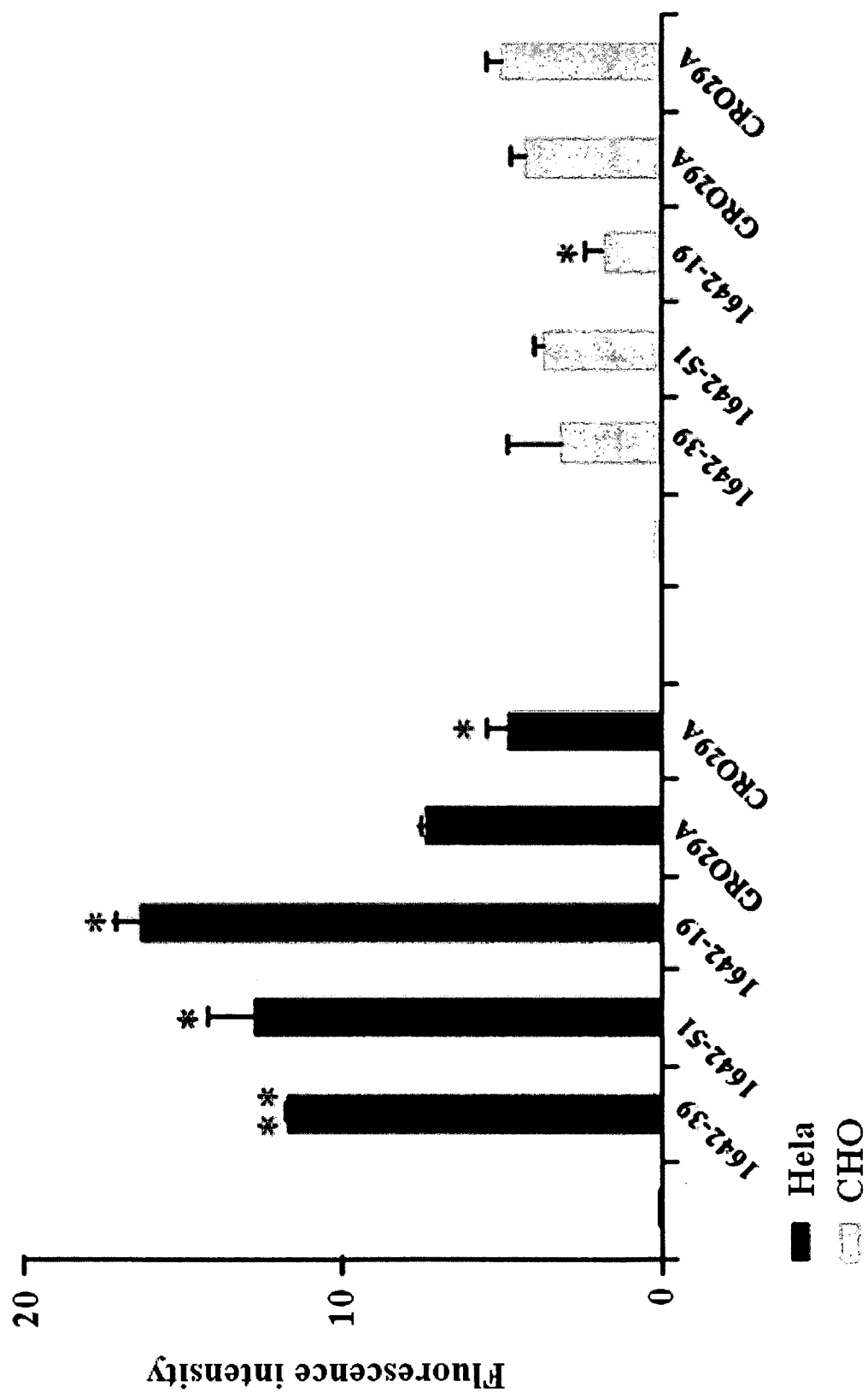
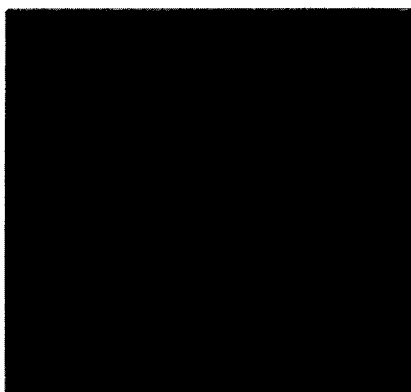
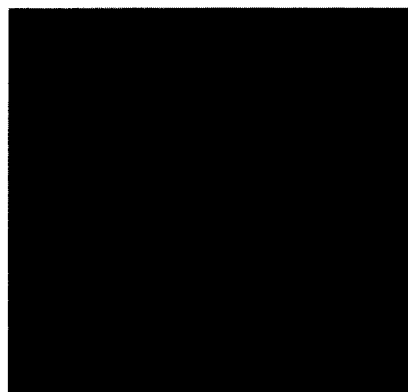


Fig. 5A

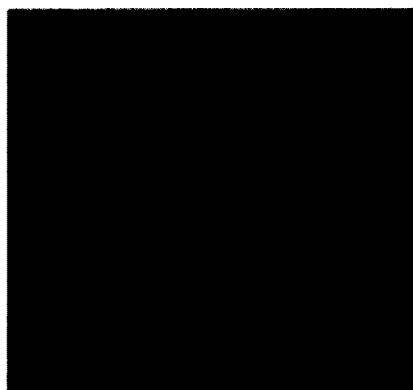
GRO29A



CRO29A



1642-39



1642-61



1642-19



HeLa cells

Fig. 5B

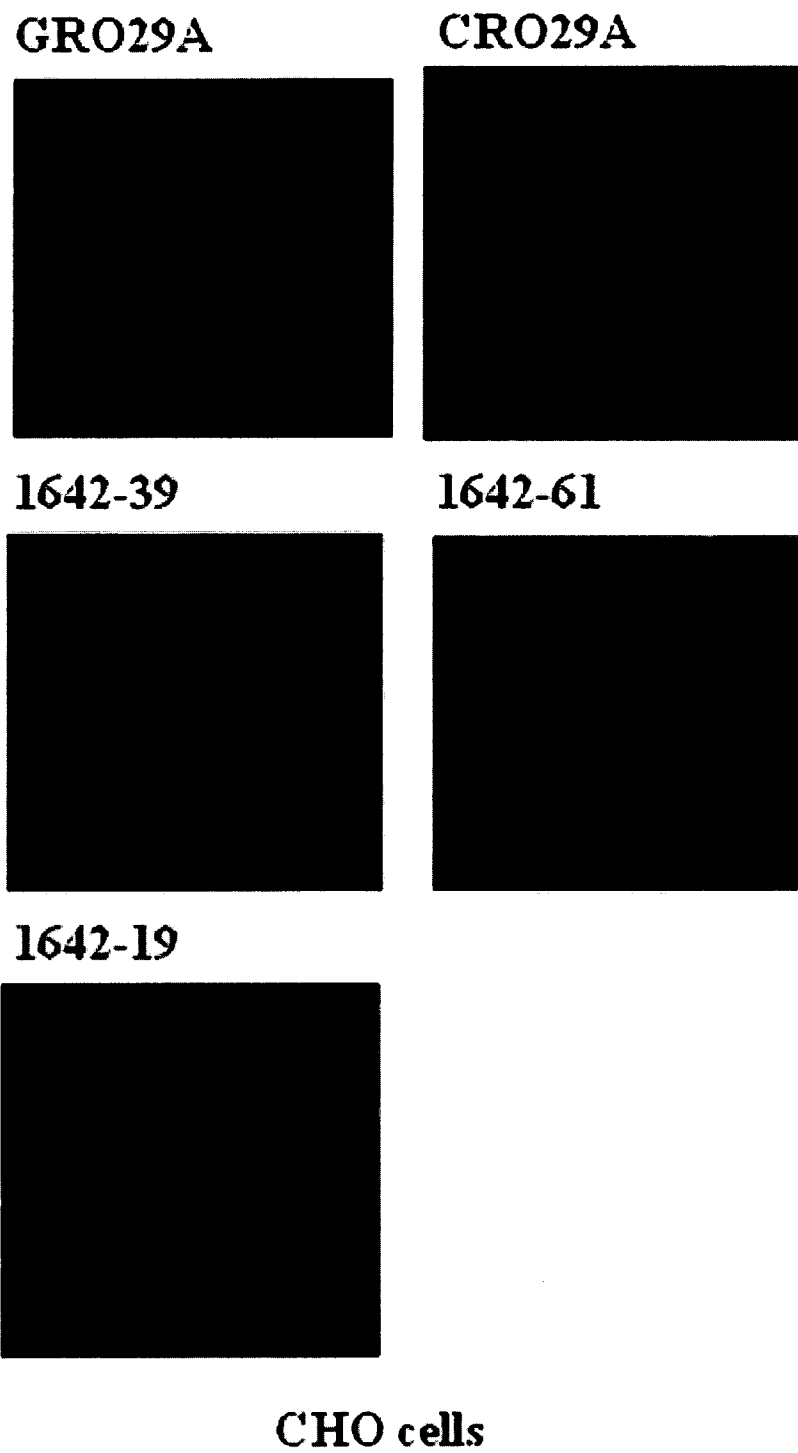


Fig. 6

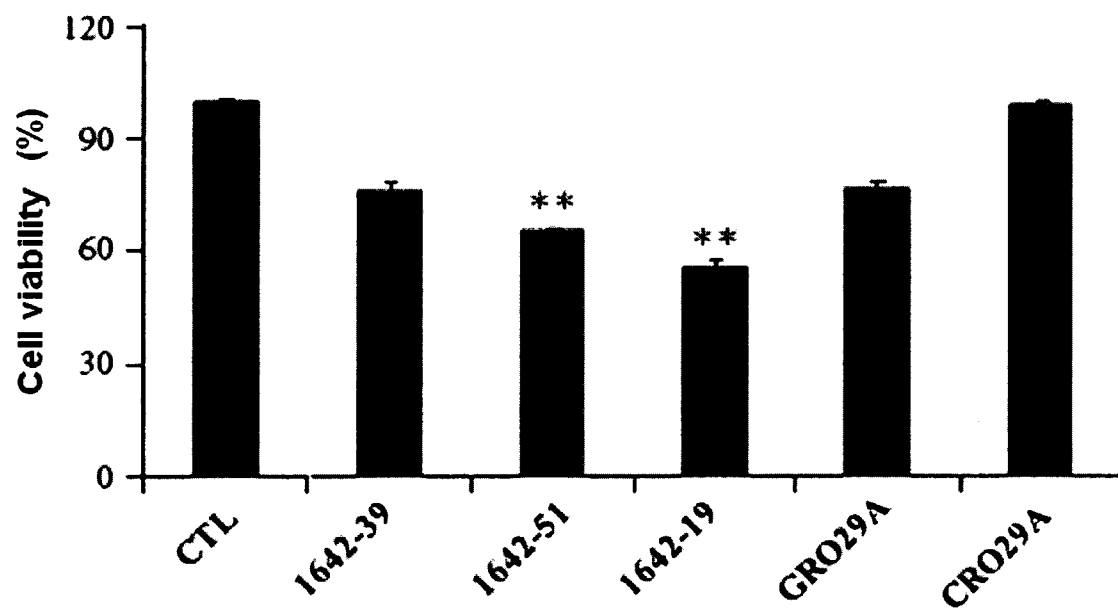


Fig. 7

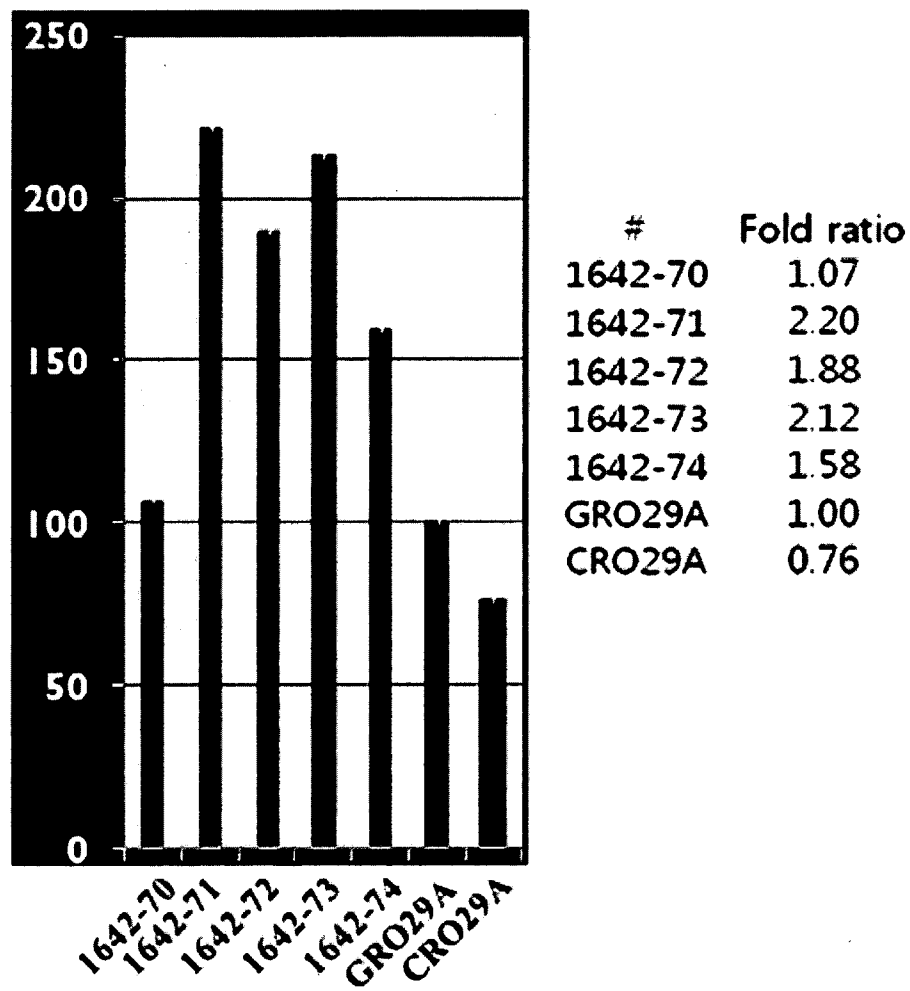


Fig. 8

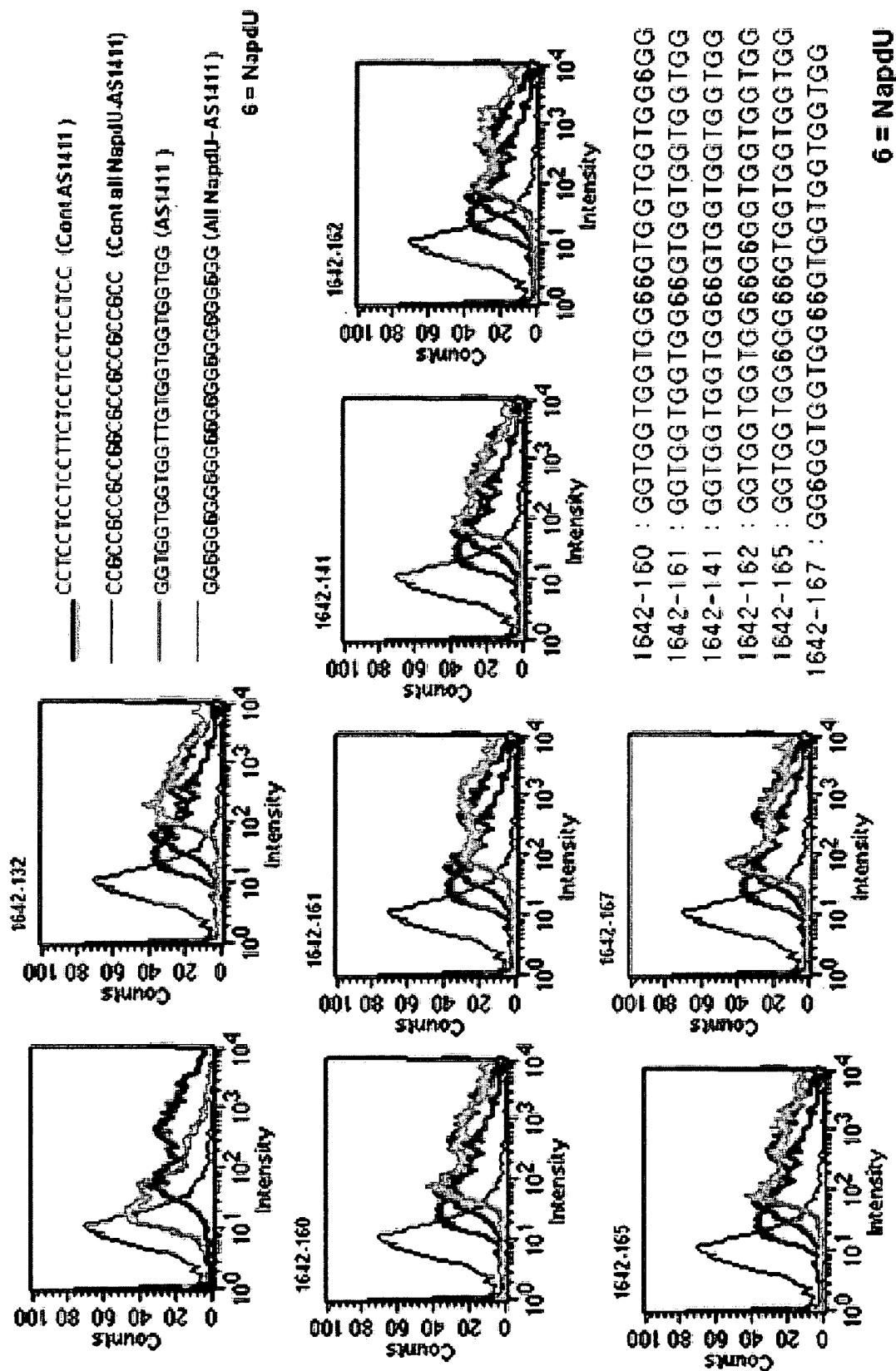


Fig. 10

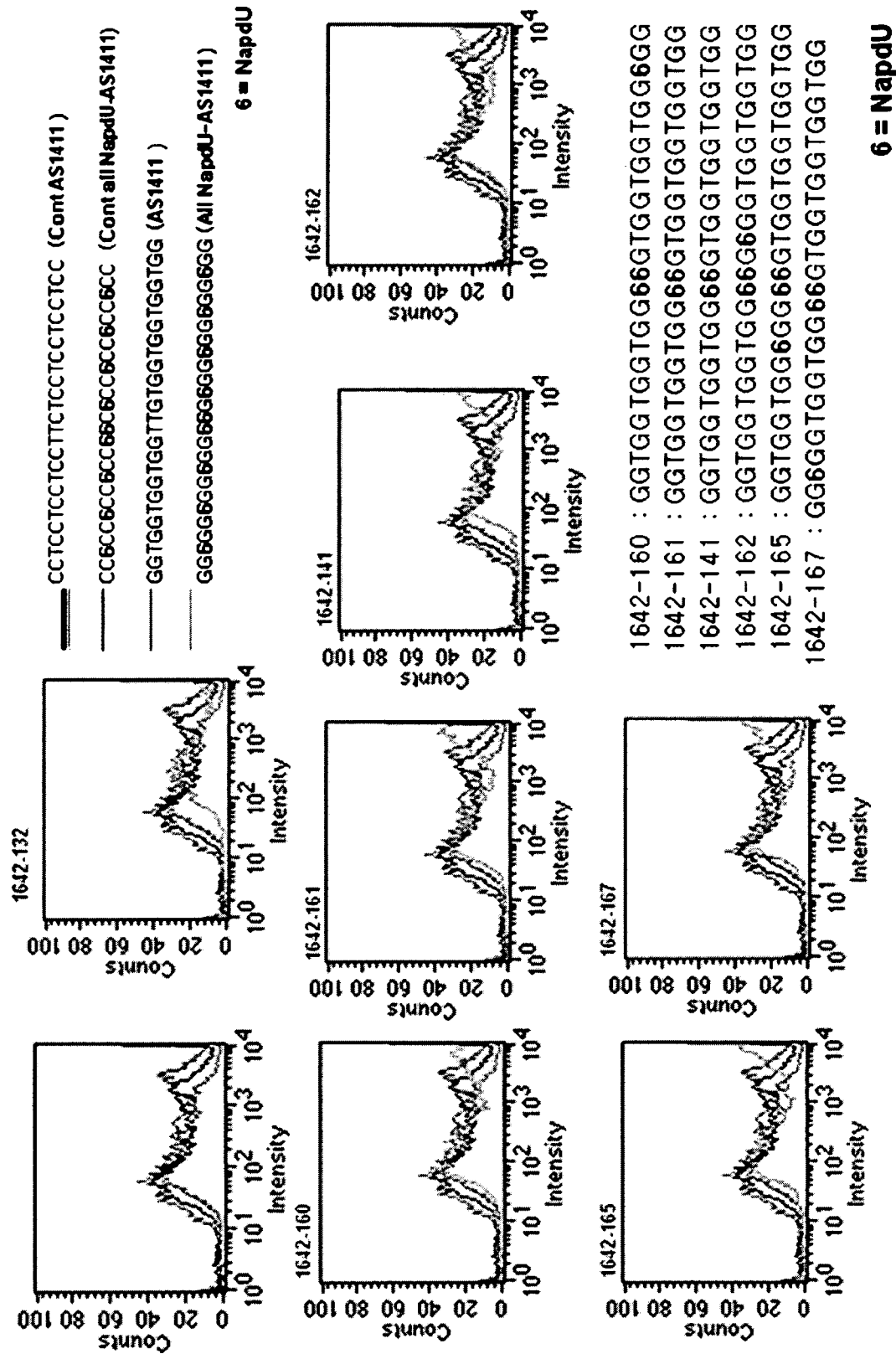


Fig. 13

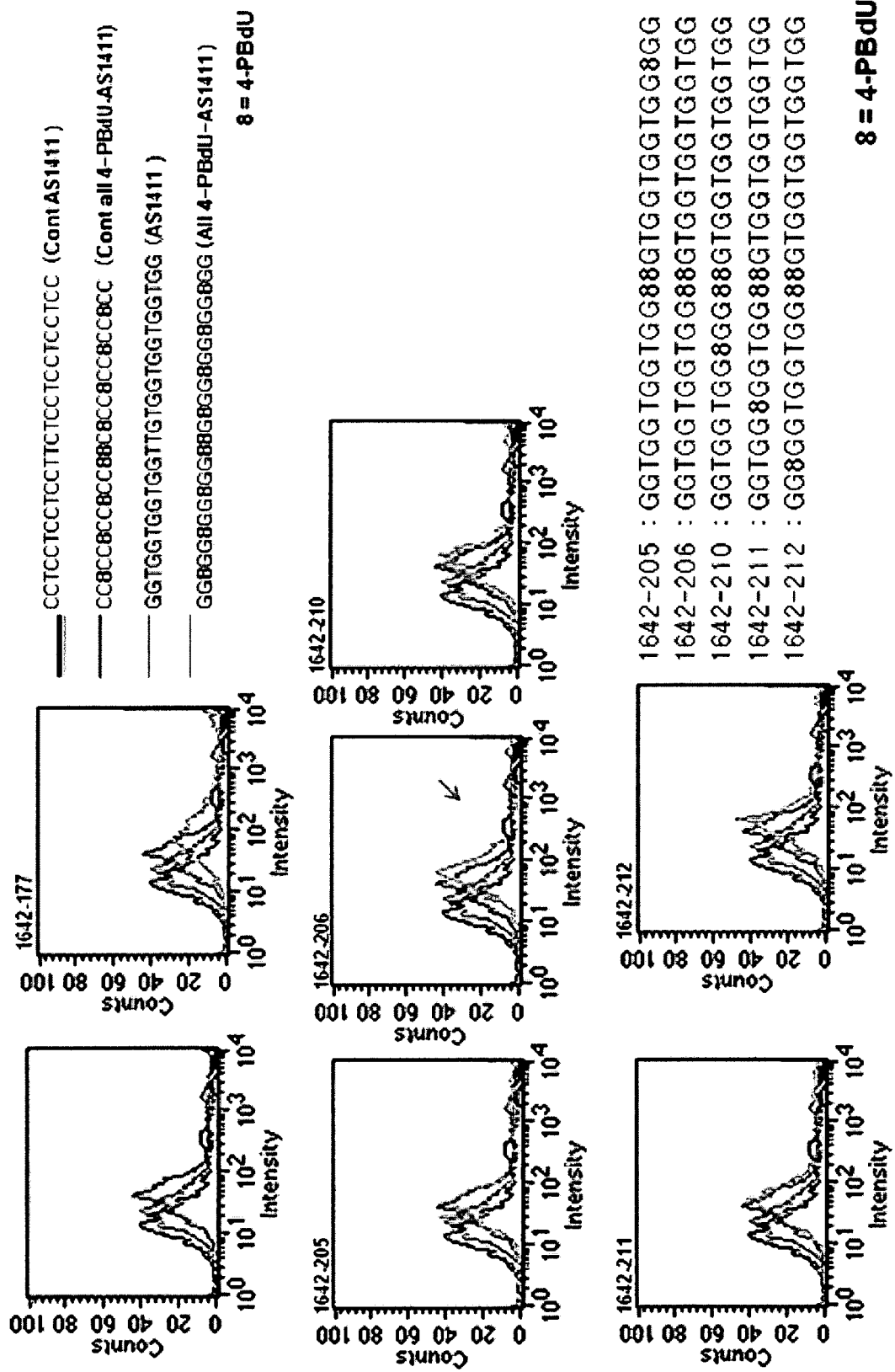


Fig. 15

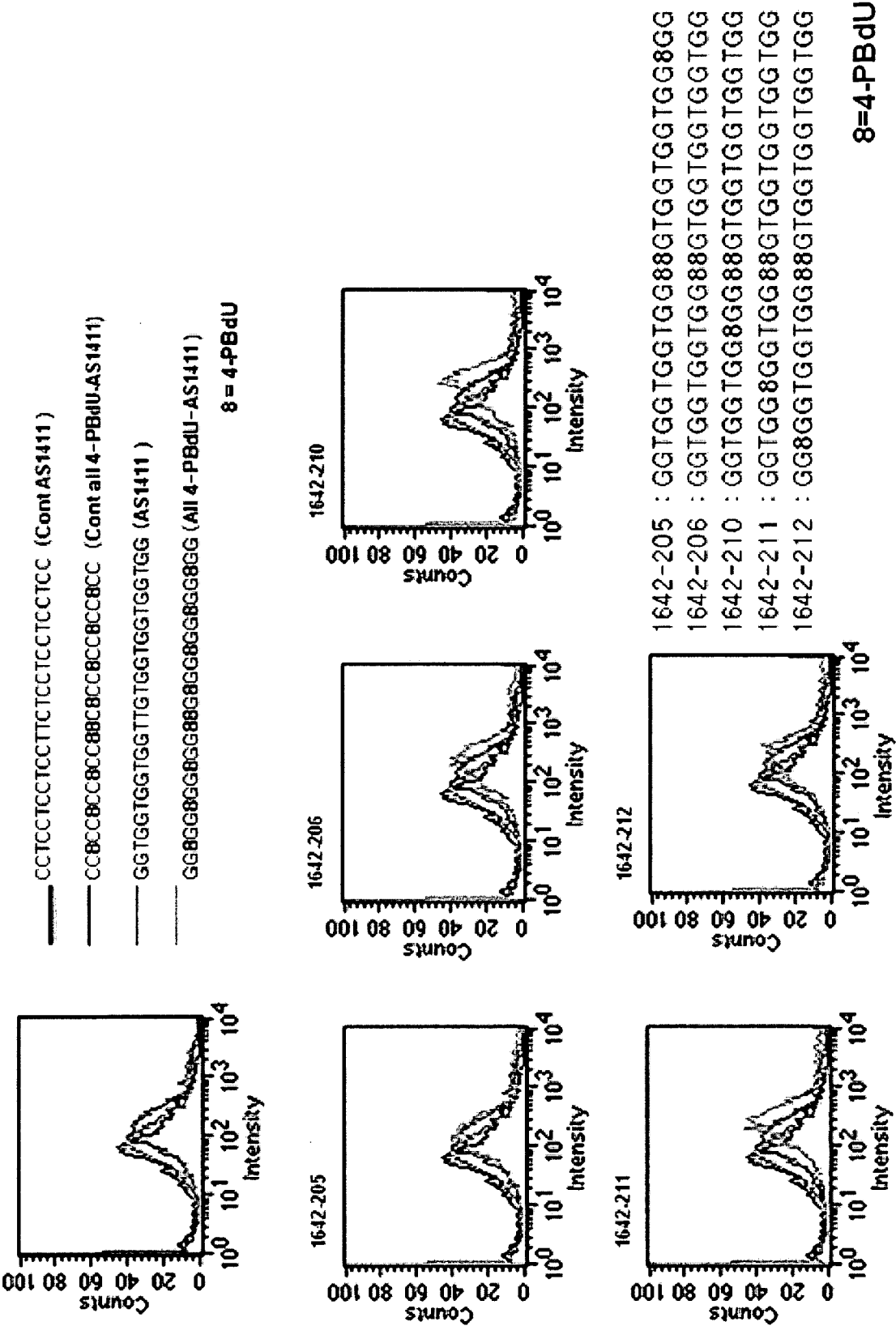


Fig. 16

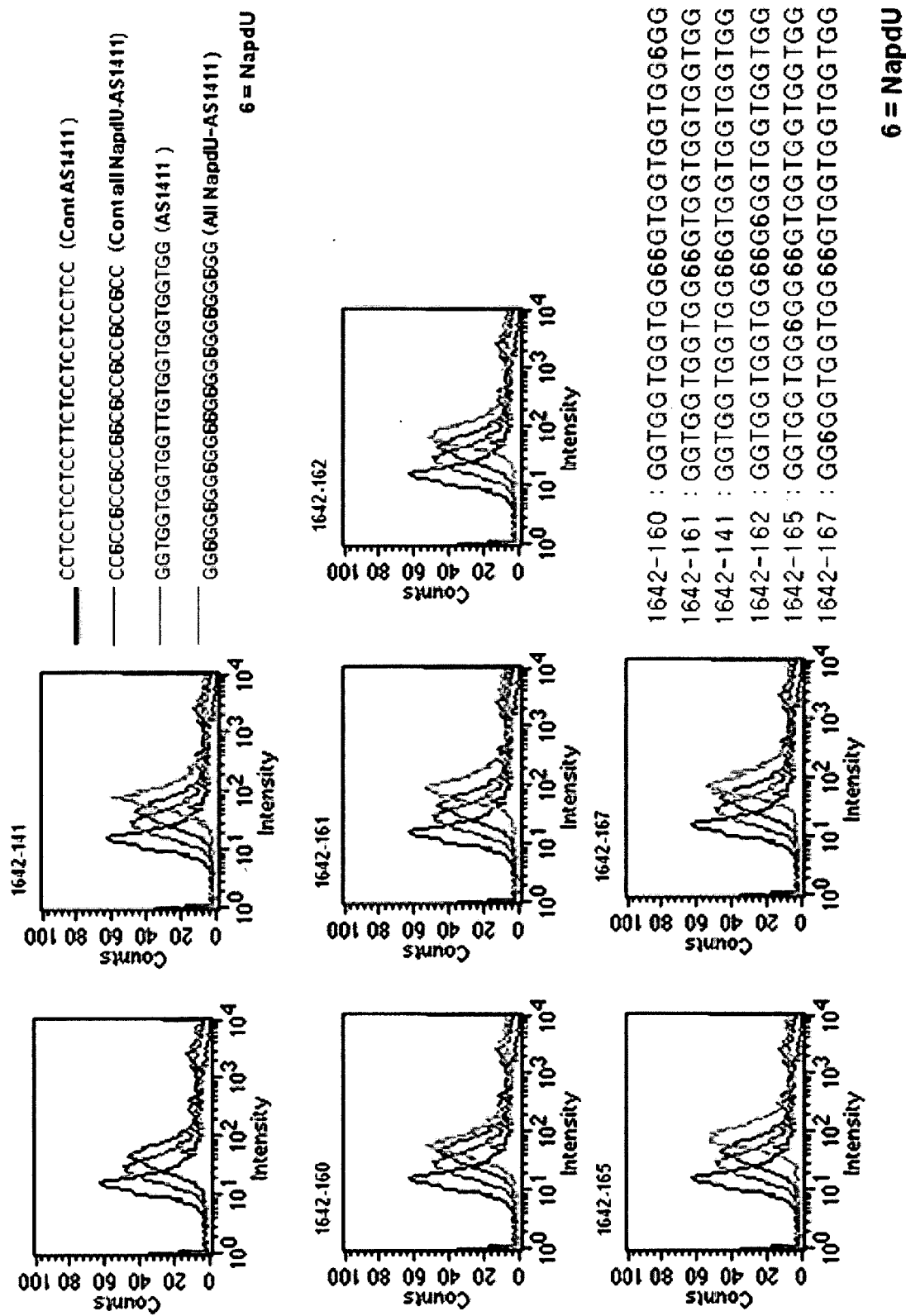


Fig. 17

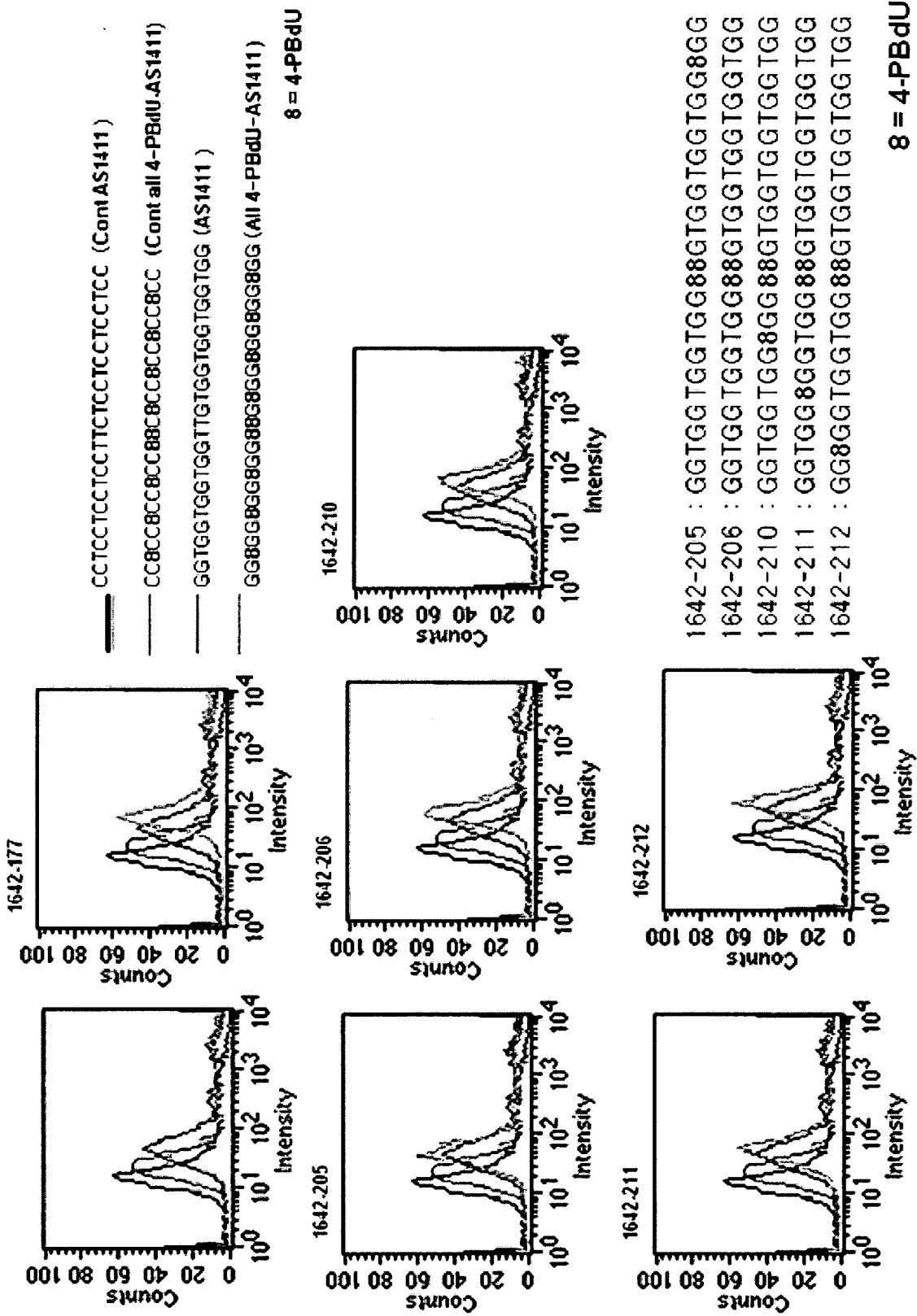


Fig. 18

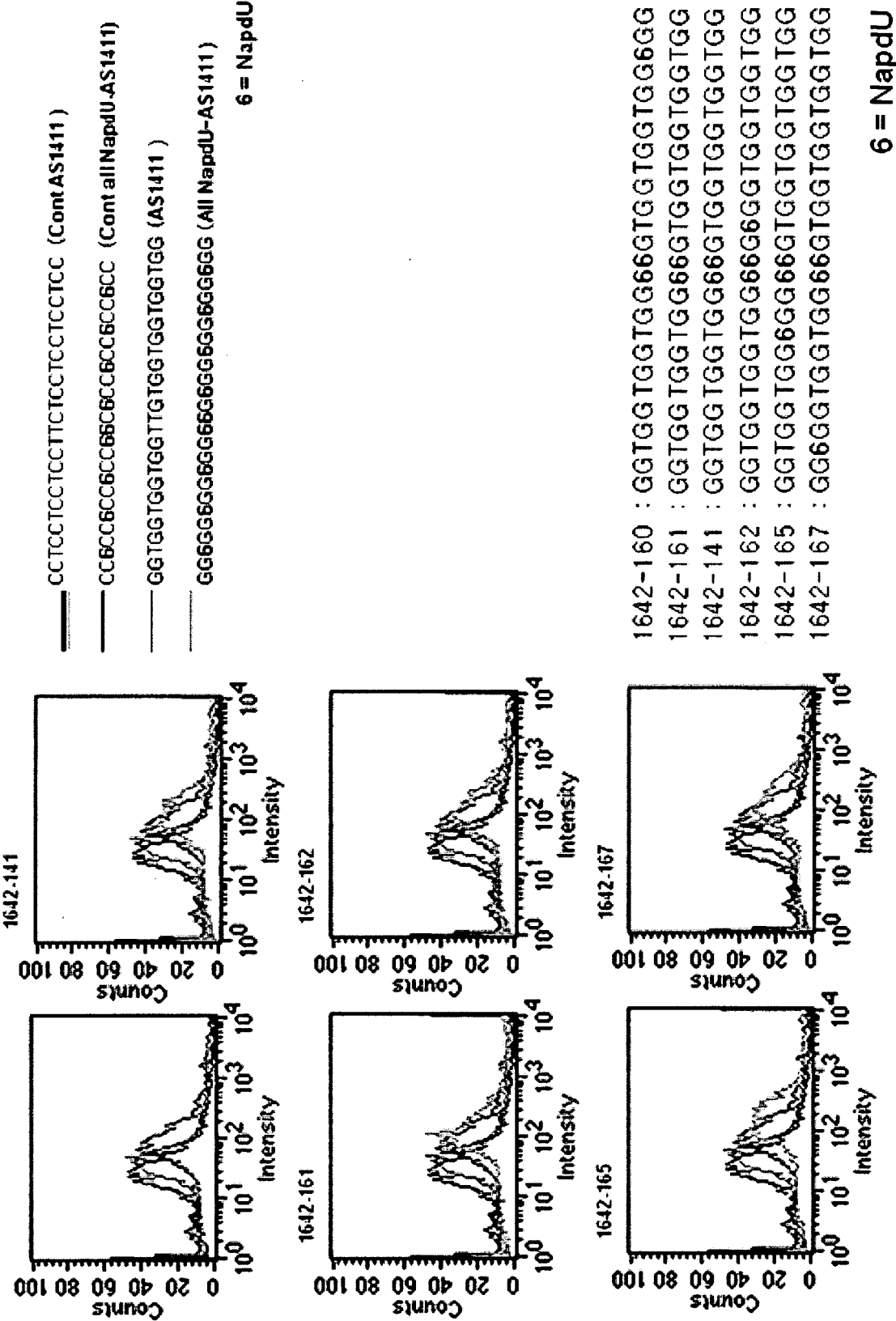


Fig. 19

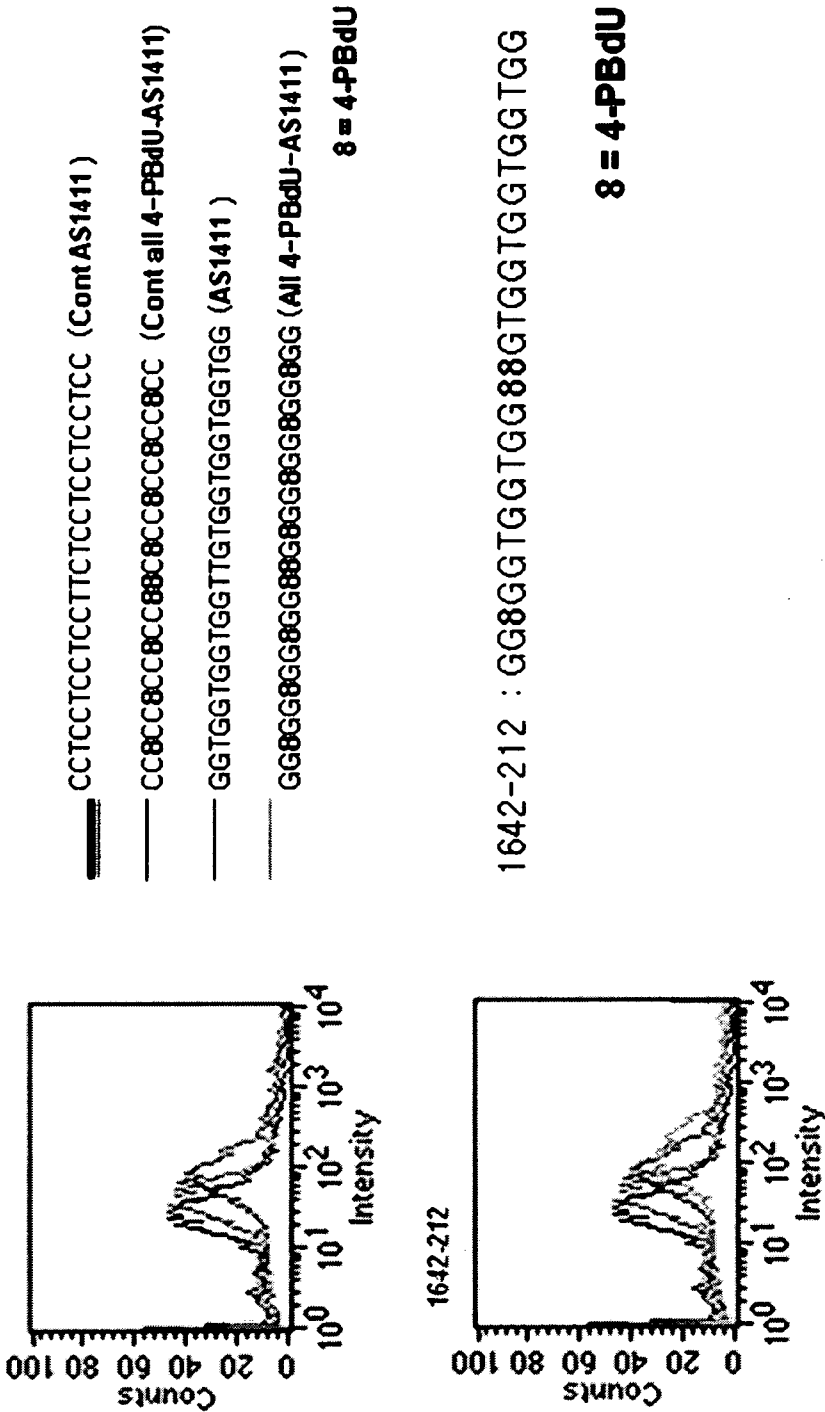


Fig. 20

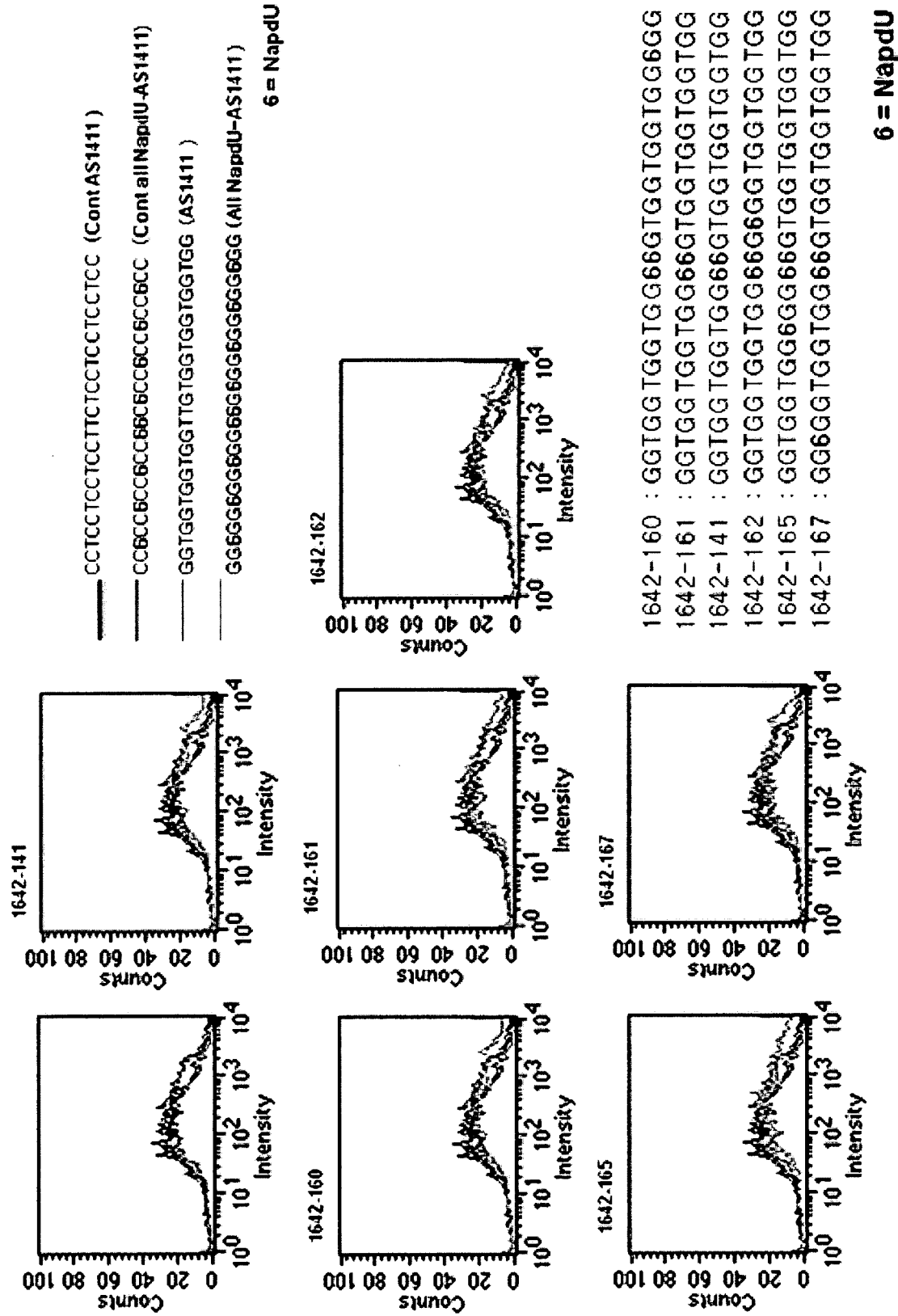


Fig. 21

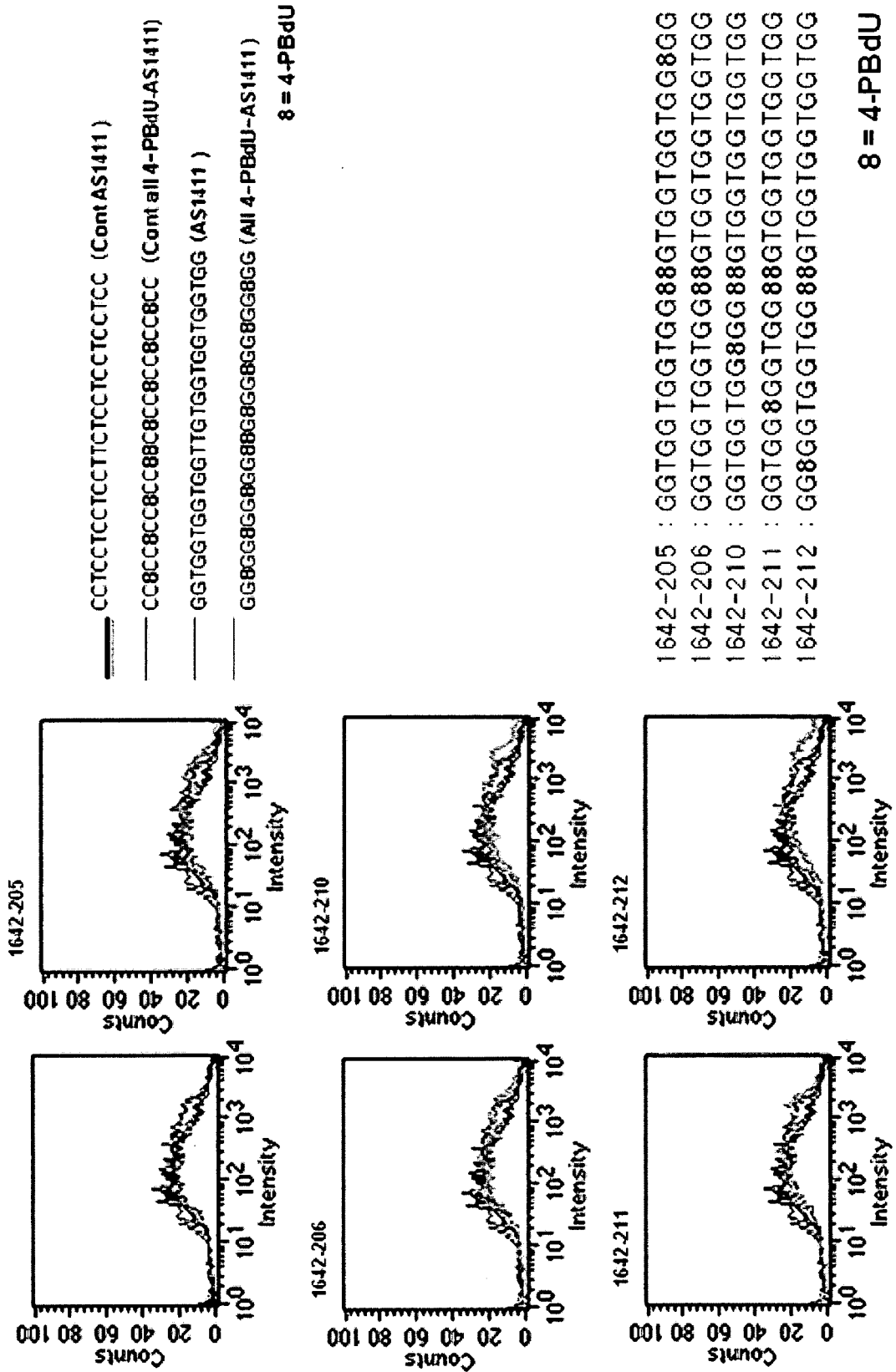


Fig. 22

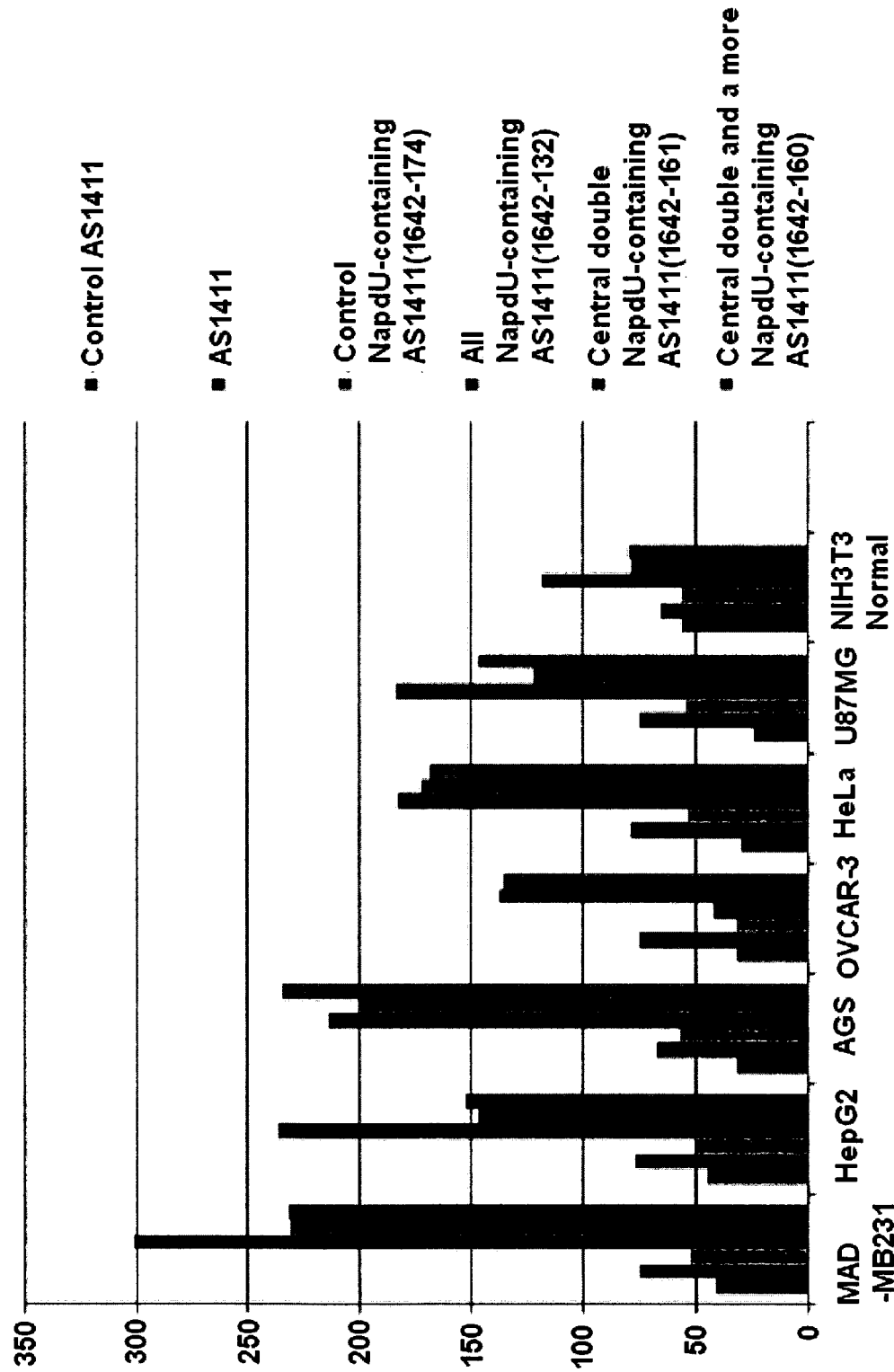


Fig. 23

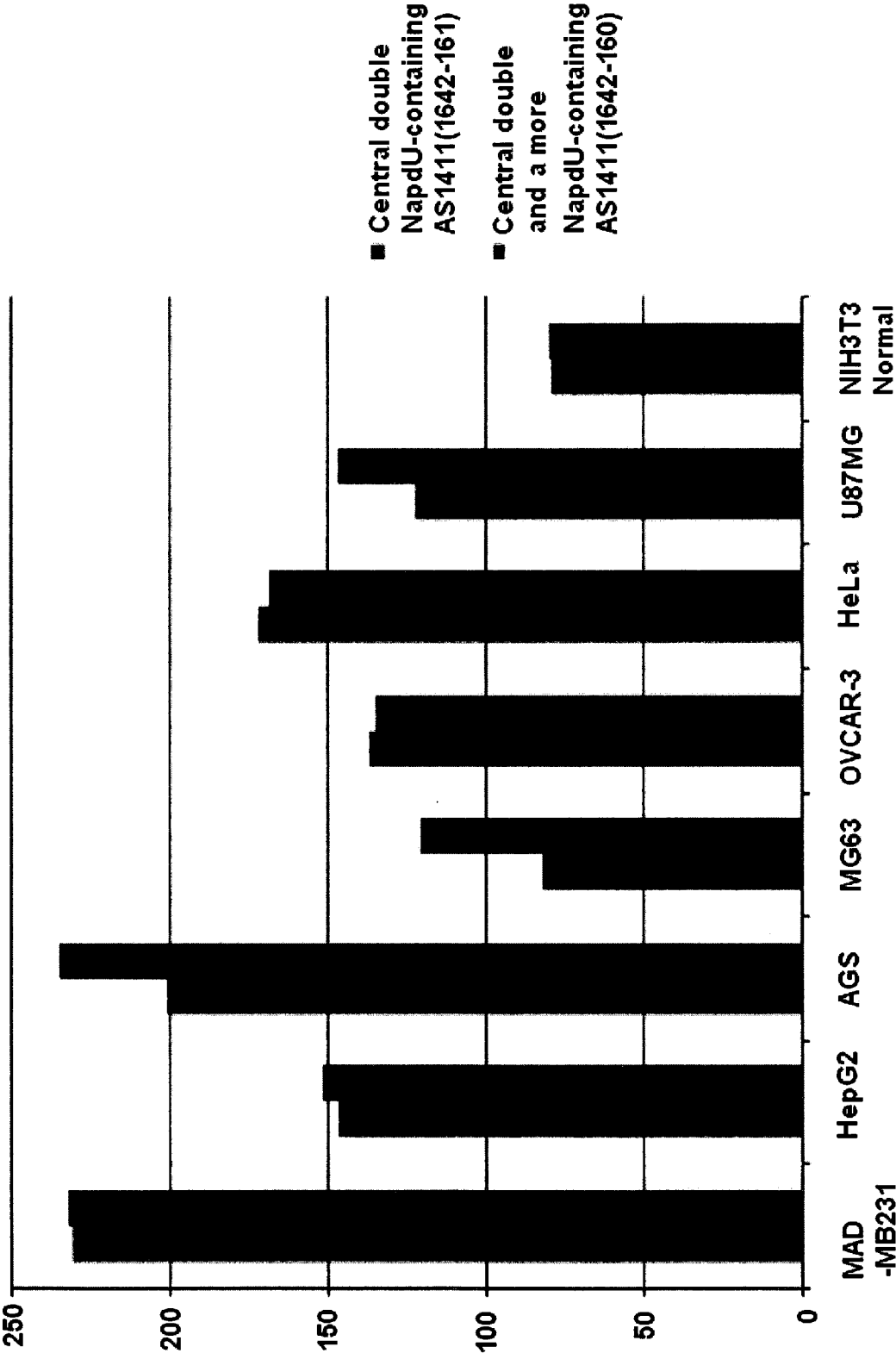


Fig. 24

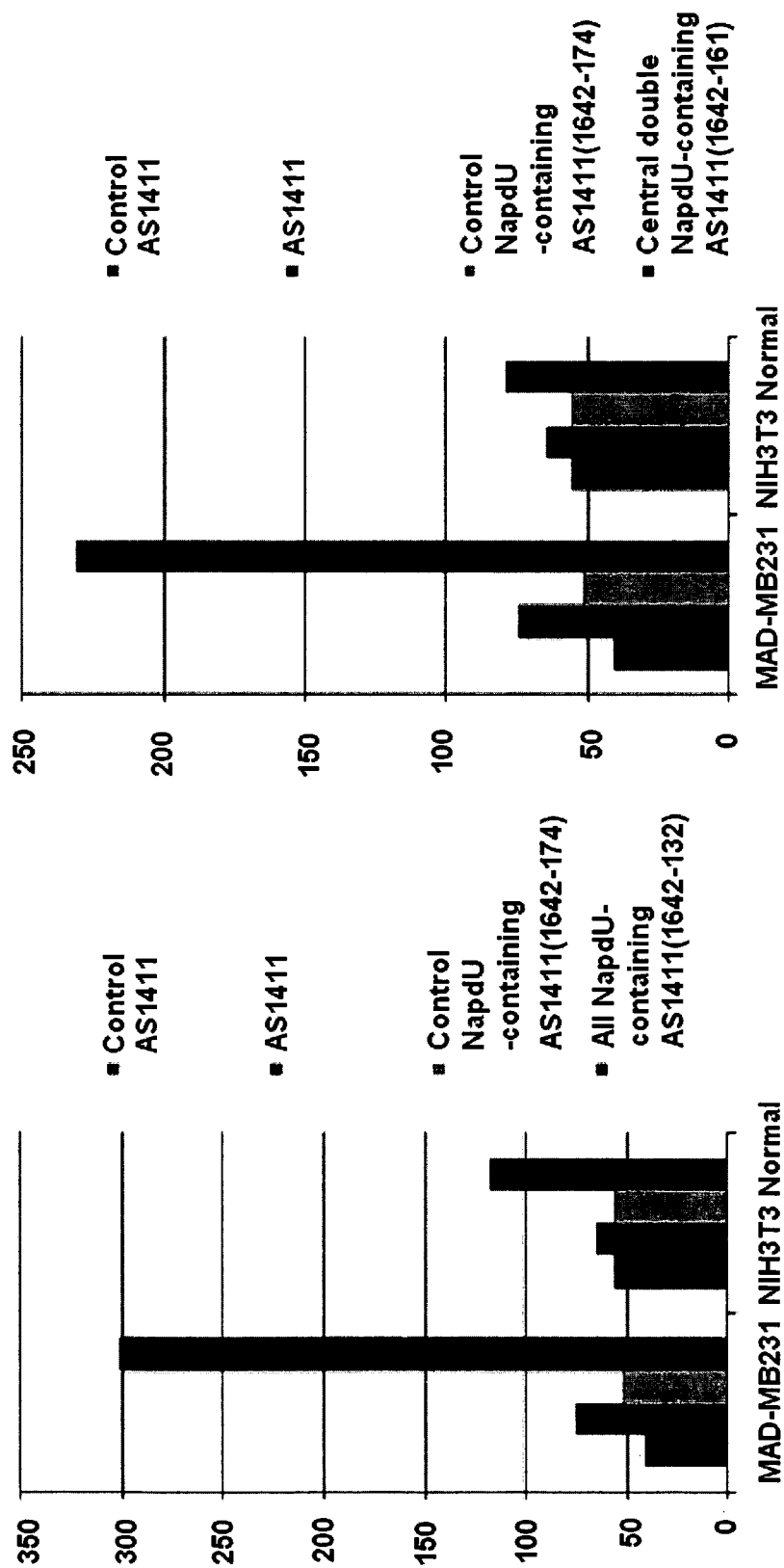


Fig. 25

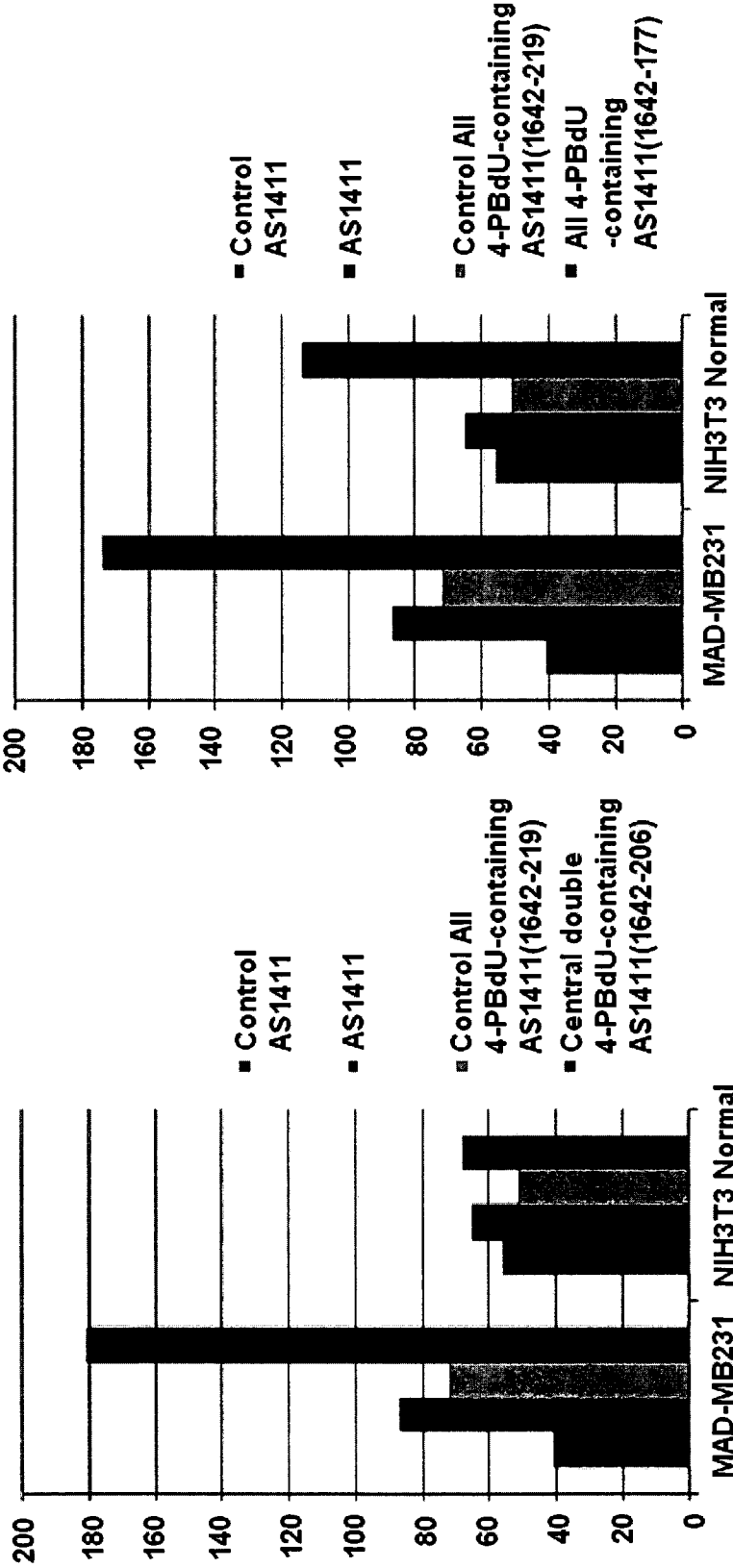


Fig. 26

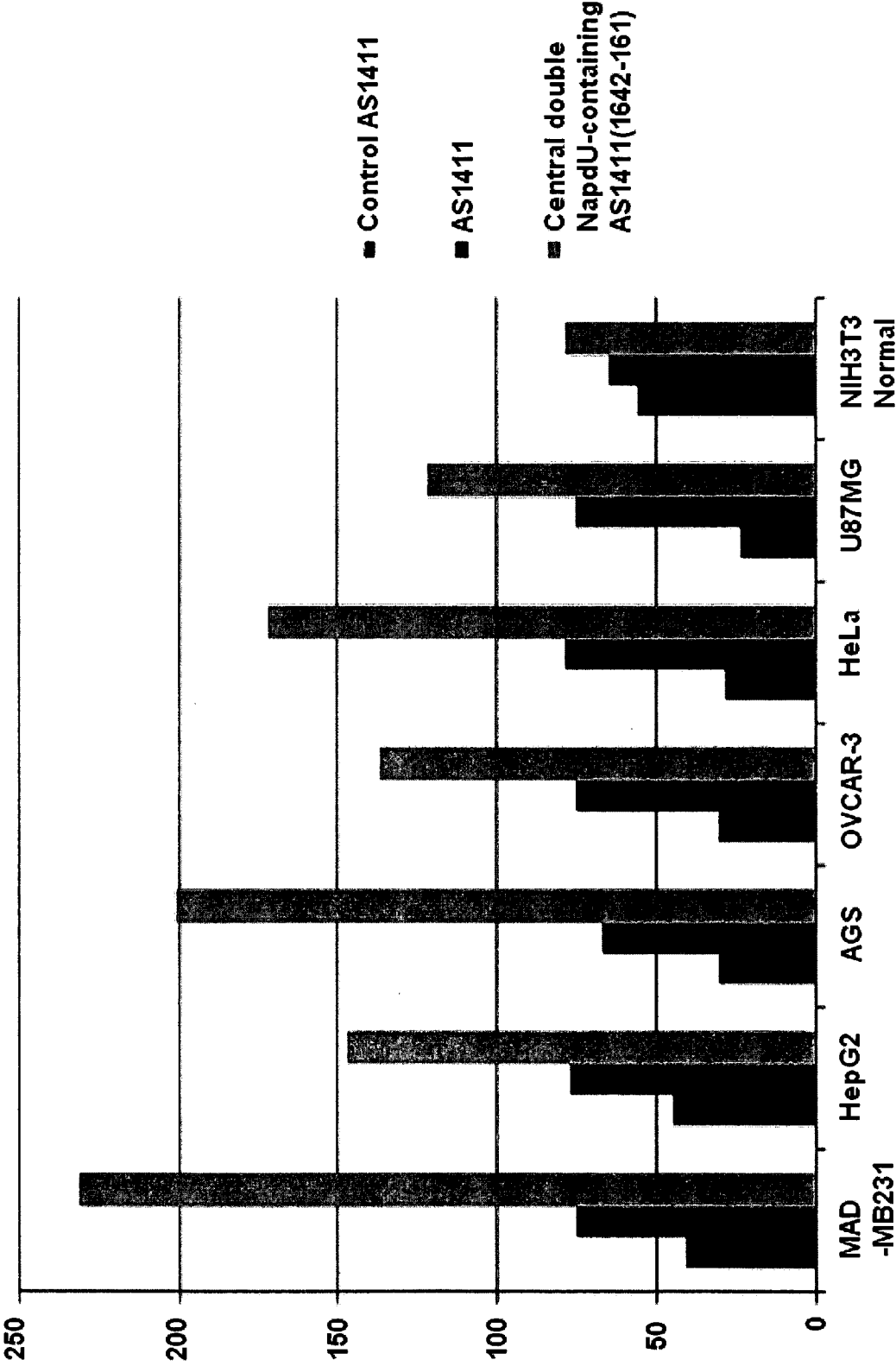


Fig. 27

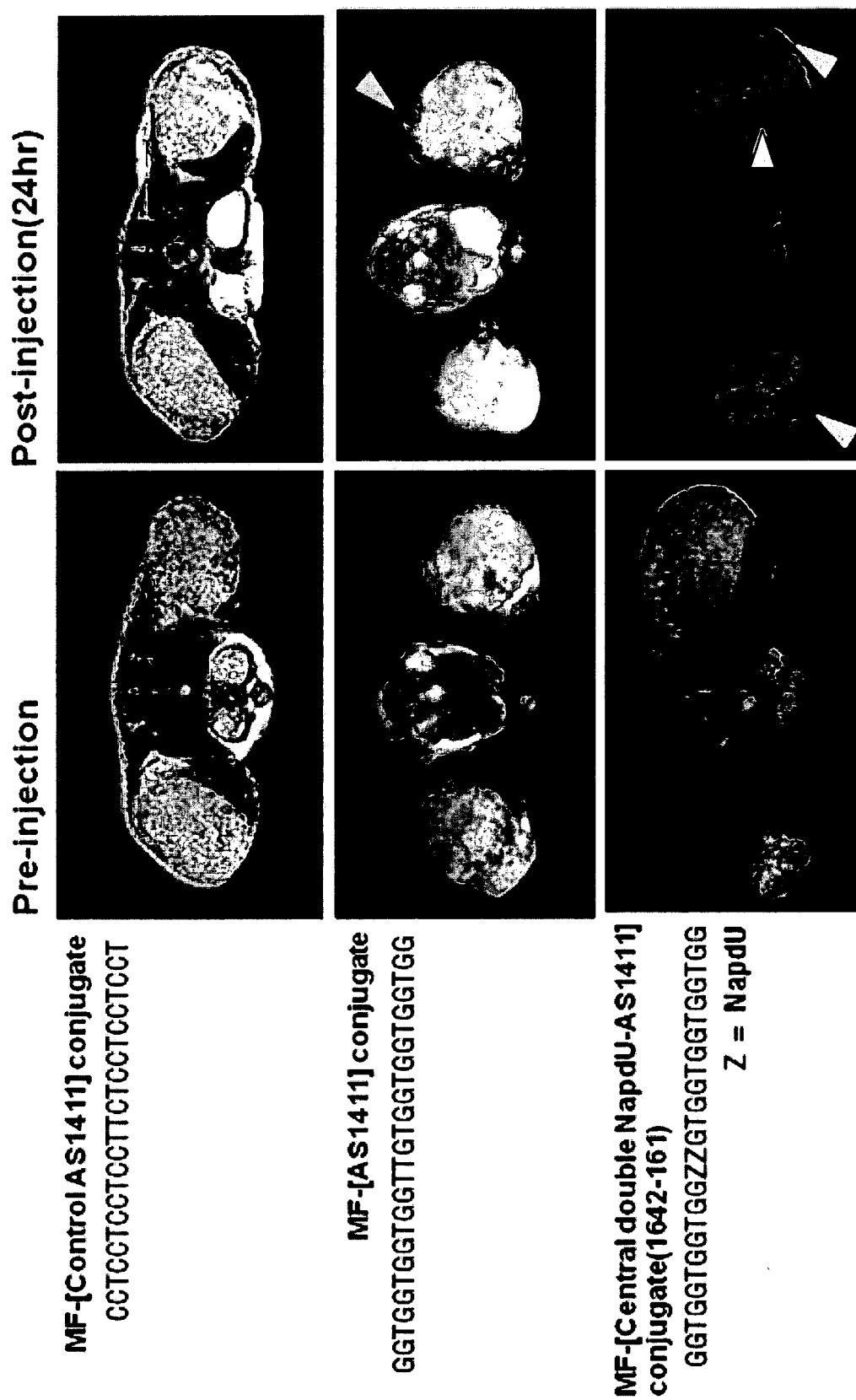
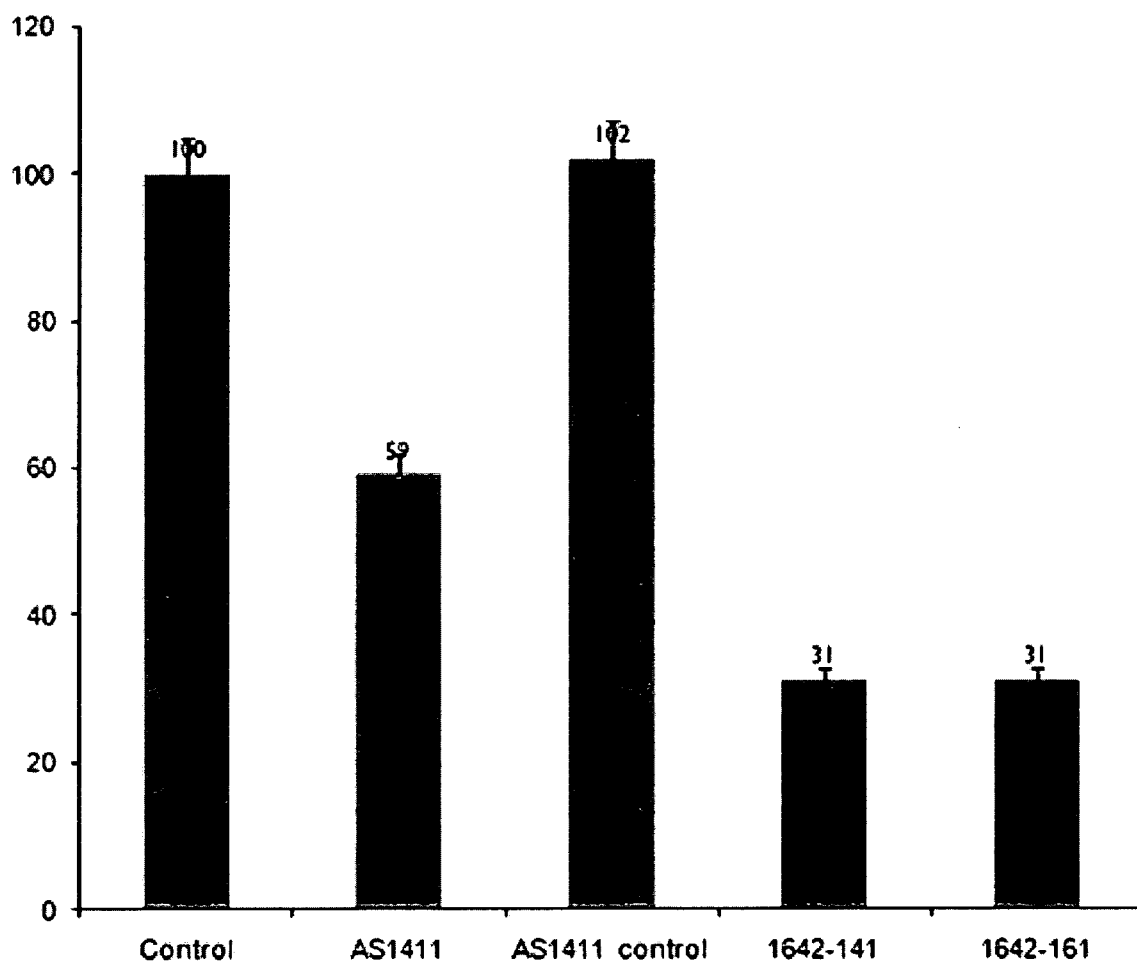
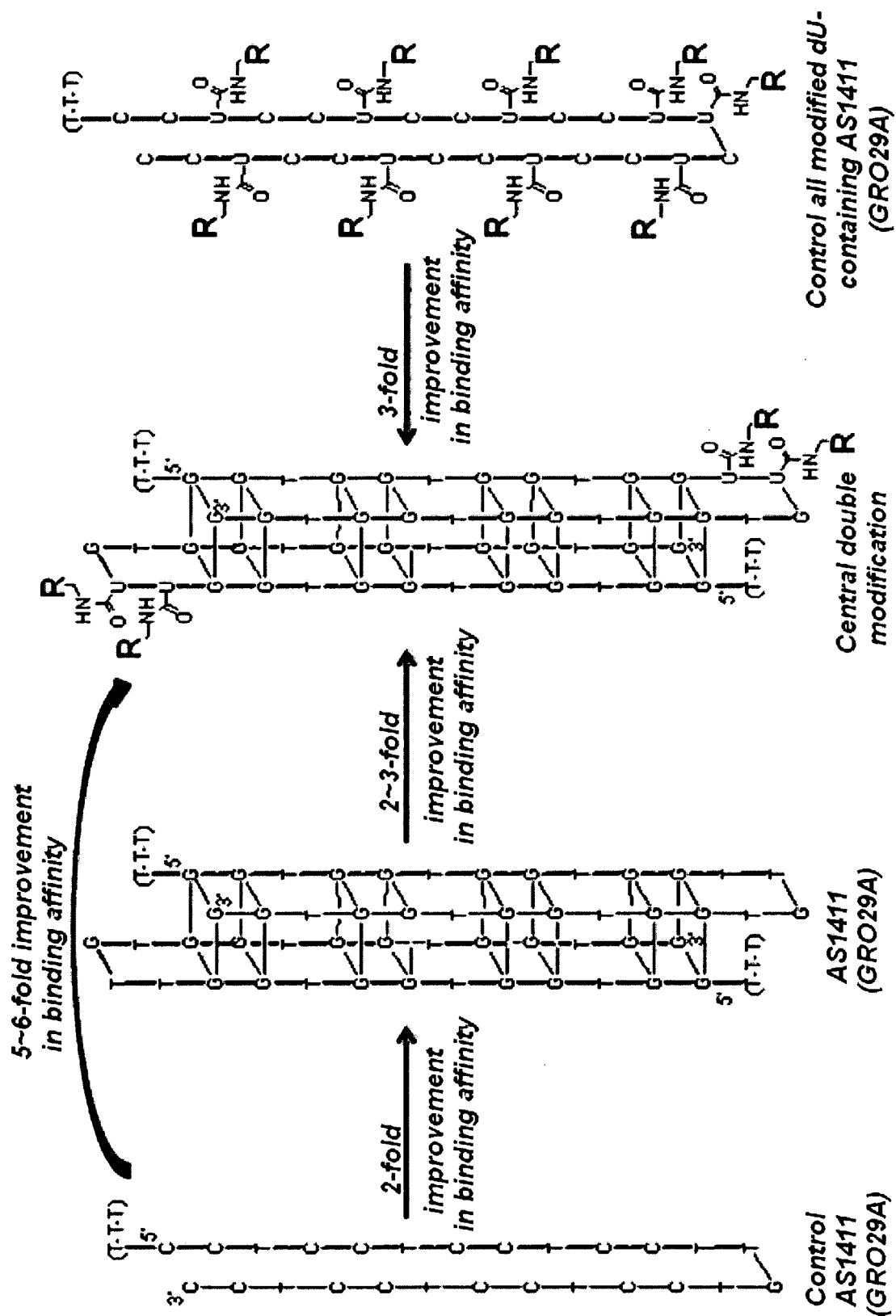


Fig. 28

Cell viability (%)

- Control : CCTCCTCCTCCTTCTCCTCCTCCT ↵
- AS1411 : GGTGGTGGTGGTGGTGGTGGTGGTGG ↵
- AS1411 control : CC6CC6CC6CC66C6CC6CC6CC6CC ↵
- 1642-141 : GGTGGTGGTGG66GTGGTGGTGGTGG ↵
- 1642-161 : GGTGGTGGTGG66GTGGTGGTGG6GG ↵

Fig. 29



A. CLASSIFICATION OF SUBJECT MATTER*C12N 15/115(2010.01)i, C12Q 1/68(2006.01)i, G01N 33/574(2006.01)i, A61K 31/7088(2006.01)i, A61P 35/00(2006.01)i*

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N 15/115

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKOMPASS(KIPO internal), Google, PubMed

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LEE, K. Y. et al., Bioimaging of nucleolin aptamer-containing 5-(N-benzylcarboxamide)-2'-deoxyuridine more capable of specific binding to targets in cancer cells, J Biomed. Biotechnol., Epub 1 Mar. 2010, Article ID 168306, 9 pages See figs. 1-5 and table 1	1-14
A	SOUNDARARAJAN, S. et al., Plasma Membrane Nucleolin Is a Receptor for the Anticancer Aptamer AS1411 in MV4-11 Leukemia Cells, Molecular Pharmacology, November 2009, Vol. 76, No. 5, pp. 984-991 See abstract	1-14
A	SOUNDARARAJAN, S. et al., The Nucleolin Targeting Aptamer AS1411 Destabilizes Bcl-2 Messenger RNA in Human Breast Cancer Cells, Cancer Res., 1 April 2008, Vol. 68, No. 7, pp. 2358-2365 See abstract	1-14
A	CHOI, E. W. et al., Cancer-selective antiproliferative activity is a general property of some G-rich oligodeoxynucleotides, Nucl. Acids Res., Mar. 2010, Vol. 38, No. 5, pp. 1623-1635. See abstract	1-14



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

14 JUNE 2011 (14.06.2011)

Date of mailing of the international search report

15 JUNE 2011 (15.06.2011)

Name and mailing address of the ISA/KR

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Seo-gu, Daejeon 302-701, Republic of Korea

Facsimile No. 82-42-472-7140

Authorized officer

Park, Jung Min

Telephone No. 82-42-481-8291



INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR2010/006311

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of :

a. a sequence listing filed or furnished

☐

on paper

☒

in electronic form

b. time of filing or furnishing

☒

contained in the international application as filed

☐

filed together with the international application in electronic form

☐

furnished subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR2010/006311Patent document
cited in search reportPublication
datePatent family
member(s)Publication
date

None