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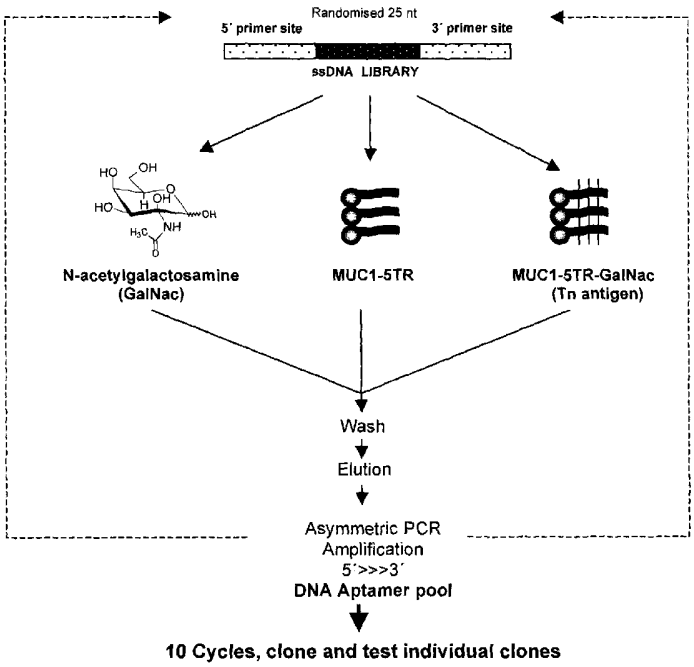
(54) **Title:** APTAMERS THAT RECOGNIZE THE CARBOHYDRATE N-ACETYLGALACTOSAMINE (GALNAC)



(57) **Abstract:** Novel oligonucleotides, termed aptamers, that bind to GalNac of the Tn antigen. In particular, aptamers that recognize the Tn antigen on a cancer cell or underglycosylated protein are disclosed. Uses of the aptamers to detect, monitor or treat cancer are also described.

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TITLE: Aptamers that Recognize the Carbohydrate N-acetylgalactosamine (GalNac)

FIELD OF THE INVENTION

The invention relates to aptamers that recognize the carbohydrate N-acetylgalactosamine (GalNac) of the Tn antigen. In particular, the invention relates to aptamers that recognize GalNac or the Tn antigen on the surface of a cancer cell and uses thereof to diagnose, monitor and treat cancer.

BACKGROUND OF THE INVENTION

Mucins are high molecular weight glycoproteins which are expressed on the surface of normal and cancer tissues. The epithelial cell mucin encoded by the MUC1 gene, is commonly expressed by adenocarcinomas of the pancreas, breast, ovary as well as by several other tumors of ductal epithelial cell origin. The protein core of mucins contains a variable number of tandem repeats (VNTRs) rich in O-glycosylation sites. The aberrant glycosylation of mucin tandem repeats by malignant cells results in the display of unique tumor-associated structures on their surface (Apostolopoulos et al. 1994; Taylor-Papadimitriou et al. 1997; Miles et al. 1999; Taylor-Papadimitriou et al. 1999). In particular, mammary epithelial cells express mucins harbouring 40 to 80 copies of the MUC1 tandem repeat, a 20-amino acid long domain with the sequence Pro-Asp-Thr-Arg-Pro-Ala-Pro-Gly-Ser-Thr-Ala-Pro-Pro-Ala-His-Gly-Val-Thr-Ser-Ala (SEQ ID NO:1) (Gendler et al. 1990; Ligtenberg et al. 1990; Wreshner et al. 1990; Haraveuni et al. 1990). All threonines and serines present in the MUC1 tandem repeat are normally O-glycosylated in the context of milk-derived mucin (Muller et al. 1997), a process initiated by at least four distinct human UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferases (Bennett et al. 1998; Wandall et al. 1997). A comprehensive analysis of 56 monoclonal antibodies that bind to the tumor-associated MUC1 mucin has revealed that a majority of them recognize peptide epitopes within the sequence Ala-Pro-Asp-Thr-Arg-Pro-Ala-Pro (SEQ

ID NO:2) (Price et al. 1998 and references thereafter) suggesting that the threonine residue within this sequence is underglycosylated in malignant cells exposing this segment of the tandem repeat. In addition, mucin-specific IgMs have been detected in sera from patients with breast and ovarian cancers and have been shown to recognize this epitope (Rughetti et al. 1993; Kotera et al. 1994; Richards et al. 1998).

MUC1 has clinical relevance as it is found on the surface of many tumors and is a useful diagnostic and therapeutic tool. In addition, MUC1 is shed from the surface of many cancers and serum concentrations may be useful for detecting certain cancers and monitoring the response to cancer therapy. Expression of MUC1 is also used for micrometastatic tumor cell detection in patients with solid tumors such as breast cancer. MUC1 is also a potential target for tumor immunotherapy.

Many cancer cells contain MUC 1 proteins and other proteins on their surface that have incomplete glycosylation. This incomplete glycosylation results in a higher amount of the Tn antigen carbohydrate structure on the surface of the cancer cell.

There is a need to identify low molecular weight agents that can target cells expressing proteins that are underglycosylated. To date, clinical strategies aimed at targeting MUC1-expressing tumor cells have been limited to the use of monoclonal antibodies and related fragments in guided immunotherapies (Hughes et al., 1997; Kramer et al. 1998; Liu et al. 1996; Shinoda et al. 1998) and at developing vaccination approaches based on the MUC1 tandem repeat (Pecher et al. 1996; Graham et al. 1996; Apostolopoulos et al. 1998).

In view of the foregoing, there is a need in the art to develop small probes and drugs able to target cancer cells with the Tn antigen on the surface. Such agents would be useful diagnostic and therapeutic tools.

SUMMARY OF THE INVENTION

The present inventors have isolated several short oligonucleotides termed aptamers (Brody and Gold, 2000) that bind N-acetylgalactosamine (GalNac), which is the carbohydrate structure of the Tn antigen. These

aptamers offer significant advantages over existing antibody-based approaches in that they display higher binding affinities to the their target, reduced immunogenicity and increased tumour penetration in view of their relatively small size. In addition, they are inexpensive to produce and represent powerful tools to target and profile different cancer tissues.

Accordingly, the present invention provides isolated aptamers that bind to GalNac of the Tn antigen. In one embodiment, the Tn antigen is on the surface of a cancer cell. In another embodiment, the aptamer binds to a sugar on a protein on the surface of a cancer cell. Any protein harboring putative O-glycosylation sites and expressed in cancer cells may present Tn antigens. Such proteins may include mucin glycoproteins, such as MUC1, MUC2 and MUC5. Jan E Hansen et al. [(1998) NetOglyc: Prediction of mucin type O-glycosylation sites based on sequence context and surface accessibility. Glycoconjugate Journal 15, 115-130] and their WEB base program at http://www.cbs.dtu.dk/services/NetOGlyc/ allows a person skilled in the art how to predict O-glycosylation sites in proteins (thus proteins that may harbor Tn antigens).

In one embodiment, the aptamer comprises all or part of the following sequences:

AGACTTAGGTGGATGTAGGATCCTT (SEQ ID NO:3);
GAACTTAGGTGGATGTAGGATCCTT (SEQ ID NO:4);
AGACTTAGGTGGATGTAGGATCCAA (SEQ ID NO:5);
GGACTTAGGTGGATGTAGGATCCTT (SEQ ID NO:6);
CTCCGATCCACTAGTAACGGCCGCC (SEQ ID NO:7);
GACCGATCCACTAGTAACGGCCGCC (SEQ ID NO:8);
AGCCGATCCACTAGTAACGGCCGCC (SEQ ID NO:9);
AACCGATCCACTAGTAACGGCCGCC (SEQ ID NO:10);
TACCGATCCACTAGTAACGGCCGCC (SEQ ID NO:11);
GACCGATCCACTAGAAACGGCCGCC (SEQ ID NO:12);
GACCGTACCACTAGTAACGGCCGCC (SEQ ID NO:13);
AAGGGATGACAGGATACGCCAAGCT (SEQ ID NO:14);
AACGCAGTACAGGATACGCCAAGCT (SEQ ID NO:15);
AACCCATGACAGGATACGCCAAGCT (SEQ ID NO:16);
TGCGGATGACAGGATACGCCAAGCT (SEQ ID NO:17); or

TAGGCTAGACAGGATACGCCAAGCT(SEQ ID NO:18).

The present invention also provides the use of the aptamers that bind GalNac of the Tn antigen in medicine. The invention provides the use of these aptamers to detect, monitor or treat cancer. The invention also includes methods of treating cancer in a mammal in need thereof comprising administering to the mammal an aptamer of the invention.

Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

Embodiments of the invention will now be described in relation to the drawings in which:

Figure 1 shows N-acetylgalactosamine (GalNac of the Tn antigen) attached to a plate.

Figure 2 shows a schematic representation of the selection process leading to the discovery of mucin MUC1-specific DNA aptamers. Single strand oligonucleotides specific for the deglycosylated MUC1 peptide tandem repeat (MUC1-5TR), its *O*-glycosylated variant harbouring up to 15 GalNac groups (Tn antigen) or *N*-acetylgalactosamine (GalNac) alone were derived using the SELEX (systematic evolution of ligands by exponential enrichment) approach (Tuerk and Gold 1990). Specifically, a pool of ssDNAs with a 25nt random region was incubated with either His-tagged MUC1-5TR peptide or its GalNac-modified variant (Tn antigen) bound to nickel-NTA beads or with N-acetylgalactosamine immobilized in wells of a 96-well plate. Bound oligonucleotides were recovered using a high salt step, desalted and amplified by asymmetric PCR before a new cycle of selection. After ten rounds of selection, each DNA aptamer pool was cloned and sequenced. Individual aptamers were synthesized and further analysed to assess their specificity.

Figure 3 shows binding specificity of DNA aptamers to their respective target. The association of 11 distinct DNA aptamers recognizing MUC1 peptide constructs (MUC1-5TR, Tn antigen) as well as the carbohydrate N-acetylgalactosamine (GalNac) with their respective target (Table 1) was confirmed using an electrophoretic mobility shift assay (EMSA). Aptamers were incubated with their target for 30 minutes and resolved on a 6% polyacrylamide DNA retardation gel (Invitrogen). Lane 1, Aptamer 5TR-1; Lane 2, Aptamer 5TR-2; Lane 3, Aptamer 5TR-3; Lane 4, Aptamer 5TR-4; Lane 5, Aptamer 5TRG-1; Lane 6, Aptamer 5TRG-2; Lane 7, Aptamer 5TRG-3; Lane 8, Aptamer 5TRG-4; Lane 9, Aptamer GalNac-1; Lane 10, Aptamer GalNac-2; Lane 11, Aptamer GalNac-3. The MUC1-5TR-GalNac peptide (Tn antigen) was used to assess the binding of aptamers targeting GalNac sugars.

Figure 4 shows specific binding of DNA aptamers to MUC1 determinants on cancer cells. The cancer cell lines MCF-7 (human breast cancer), T47D (human breast cancer), PANC-1 (human pancreatic cancer) as well as CHO cells (chinese hamster ovary; negative control) were stained with rhodamine-labeled DNA aptamers and analysed by flow cytometry. Cell-type specific binding was observed for aptamers 5TR-1 (blue), 5TRG-2 (pink) and GalNac-3 (green). Cells were also stained with the mAb *Onc*M27 (recognizes the mucin MUC1 tandem repeat; shown as an interrupted black line) to confirm the expression of MUC1 determinants on cancer cells. The background autofluorescence of unstained cells is shown as a shaded area for each cell line.

Figure 5 shows selective staining of MUC1 tumour markers on cancer cells as monitored by confocal microscopy. MCF-7, T47D, PANC-1 and CHO (negative control) cells were stained with the anti-MUC1 mAb *Onc*M27 (green colour, Lanes 1) or one of three rhodamine-labelled MUC1 aptamers (in red), namely 5TR-1 binding to the MUC1 peptide tandem repeat core (Lanes 2), 5TRG-2 recognizing the peptide-GalNac Tn antigen (Lanes 3) and GalNac-3 staining *N*-acetylgalactosamine (lanes 4). Confocal (panels A,D,G,J) and phase contrast images (panels B,L,H,K) of viable cells taken 30 minutes post incubation were superimposed to emphasize the cellular location of aptamers (panels C,F,I,L).

Figure 6 shows time dependent internalization of Rhodamine labelled aptamers into MCF-7, T47D, PANC-1, and CHO cells as measured by flow cytometry. Incubations were performed at 4^0C (▲), 37^0 C with (■) or without (●) monodansyl cadaverine for periods up to 6 hours. In all panels, curves represent the time-dependent changes in relative mean fluorescence intensity for cells exposed to 5TR-1 aptamer (a), 5TRG-2 aptamer (b), GalNac-3 aptamer (c), random 25 base (GATC repeats) aptamer (d). All data points represent the average relative mean fluorescence intensity for cells exposed to fluorescent signals from sets of experiments performed in triplicate.

Figure 7 shows cell viability curves for T47D and CHO incubated (2hour) in the presence of increasing amounts of free chlorin E6 (♦), Ce6-5TR-1 aptamer (■), Ce6-5TRG-2 aptamer (▲), Ce6-GalNac-3 aptamer(●), 5TR-1 aptamer (□) , 5TRG-2 aptamer (Δ), GalNac-3 aptamer (○). Cell viability was determined for cells exposed (+) or not (-) to photoirradiation. Cell viability was reported as the percentage surviving cells measured using the MTT toxicity assay. The term concentration represents the molar concentration of Chlorin e6 either free or attached to the aptamer constructs.

Figure 8 shows a proposed mechanism of internalization. MUC1-directed aptamers (black shape) binds to membrane-bound, underglycosylated MUC1 mucin (lego-like modules with small circles). Aberrantly glycosylated mucins are recycled from the cell surface to the trans Golgi network where they are further glycosylated before returning to the cell surface.

Figure 9 shows the structure of a Chlorin e6 (Ce6) aptamer.

DETAILED DESCRIPTION OF THE INVENTION

The invention relates to aptamers that recognize N-acetylgalactosamine (also known as GalNac) of the Tn antigen. GalNac is a carbohydrate tumor marker present on epithelial cancer cells such as breast, ovarian, colon, lung, and prostate cancer cells. These aptamers recognize the sugar alone or linked to a protein (as a post-translational modification), lipid, polymer or other substrate. The aptamers are able to selectively recognize GalNac and the Tn antigen present on the apical surface of neoplastic cells allowing for the identification and targeting of the diseased

cell, by the conjugating an appropriate diagnostic (eg. fluorescence probe, biotin) or anticancer agent (eg. drug, photodynamic therapy drugs, radionuclide, toxin, cytotoxic peptide, oligonucleotide or plasmid encoding an anticancer agent) to the aptamer.

1. Aptamers of the Invention

The present inventors have isolated several novel oligonucleotides, termed aptamers that bind to GalNac of the Tn antigen, preferably on a cancer cell. Accordingly, the present invention provides isolated aptamers that bind to GalNac or to the Tn antigen. In a preferred embodiment, the Tn antigen is present on the surface of a cancer cell. The aptamers are optionally purified to at least 95%, at least 97% or at least 99% purity.

Aptamers were identified from an initial 25mer library of 4^{25} random sequences of DNA molecules using the SELEX approach (Systematic Evolution of Ligands by Exponential enrichment) for their ability to bind to the Tn antigen sugar.

Using this approach, the following aptamers were isolated:

AGACTTAGGTGGATGTAGGATCCTT (SEQ ID NO:3);
GAACTTAGGTGGATGTAGGATCCTT (SEQ ID NO:4);
AGACTTAGGTGGATGTAGGATCCAA (SEQ ID NO:5);
GGACTTAGGTGGATGTAGGATCCTT (SEQ ID NO:6);
CTCCGATCCACTAGTAACGGCCGCC (SEQ ID NO:7);
GACCGATCCACTAGTAACGGCCGCC (SEQ ID NO:8);
AGCCGATCCACTAGTAACGGCCGCC (SEQ ID NO:9);
AACCGATCCACTAGTAACGGCCGCC (SEQ ID NO:10);
TACCGATCCACTAGTAACGGCCGCC (SEQ ID NO:11);
GACCGATCCACTAGAAACGGCCGCC (SEQ ID NO:12);
GACCGTACCACTAGTAACGGCCGCC (SEQ ID NO:13);
AAGGGATGACAGGATACGCCAAGCT (SEQ ID NO:14);
AACGCAGTACAGGATACGCCAAGCT (SEQ ID NO:15);
AACCCATGACAGGATACGCCAAGCT (SEQ ID NO:16);
TGCGGATGACAGGATACGCCAAGCT (SEQ ID NO:17); and
TAGGCTAGACAGGATACGCCAAGCT (SEQ ID NO:18).

Accordingly, in one embodiment, the aptamer comprises all or part of the following sequences:

AGACTTAGGTGGATGTAGGATCCTT (SEQ ID NO:3);
GAACTTAGGTGGATGTAGGATCCTT (SEQ ID NO:4);
AGACTTAGGTGGATGTAGGATCCAA (SEQ ID NO:5);
GGACTTAGGTGGATGTAGGATCCTT (SEQ ID NO:6);
CTCCGATCCACTAGTAACGGCCGCC (SEQ ID NO:7);
GACCGATCCACTAGTAACGGCCGCC (SEQ ID NO:8);
AGCCGATCCACTAGTAACGGCCGCC (SEQ ID NO:9);
AACCGATCCACTAGTAACGGCCGCC (SEQ ID NO:10);
TACCGATCCACTAGTAACGGCCGCC (SEQ ID NO:11);
GACCGATCCACTAGAAACGGCCGCC (SEQ ID NO:12);
GACCGTACCACTAGTAACGGCCGCC (SEQ ID NO:13);
AAGGGATGACAGGATACGCCAAGCT (SEQ ID NO:14);
AACGCAGTACAGGATACGCCAAGCT (SEQ ID NO:15);
AACCCATGACAGGATACGCCAAGCT (SEQ ID NO:16);
TGCGGATGACAGGATACGCCAAGCT (SEQ ID NO:17); or
TAGGCTAGACAGGATACGCCAAGCT (SEQ ID NO:18).

Useful portions of the aforementioned sequences bind to GalNac of the Tn antigen, particularly the Tn antigen on a cancer cell. In one embodiment, the aptamer of the invention binds to an N-terminal GalNac of the Tn antigen (Hanisch 2001). In another embodiment, the aptamers of the invention bind to the Tn antigen, particularly the Tn antigen on a cancer cell. Tn antigen binding activity is readily determined against a Tn antigen sugar as described above. Uses of these aptamers are described below.

The term "aptamer" as used herein means a short oligonucleotide that can bind to an antigen. The aforementioned oligonucleotide can be at least 75, 60, 50, 40, 30, 25, 20, 15 or 10 base pairs in length. The term "oligonucleotide" includes DNA and RNA, and can be double stranded or single stranded. In one embodiment, the oligonucleotide is DNA. In a further embodiment, the oligonucleotide is single stranded DNA.

The term "Tn antigen aptamer" as used herein means a short oligonucleotide that can bind to the Tn antigen.

The invention also includes fragments, analogs and derivatives of the aptamers described herein which maintain the ability to bind GalNac or the Tn antigen. The term includes any oligomers or polymers of nucleotide or nucleoside monomers consisting of naturally occurring bases, sugars, and intersugar (backbone) linkages. The term also includes modified or substituted oligomers comprising non-naturally occurring monomers or portions thereof, which function similarly. Such modified or substituted oligonucleotides may be preferred over naturally occurring forms because of properties such as enhanced cellular uptake, or increased stability in the presence of nucleases. The term also includes chimeric oligonucleotides that contain two or more chemically distinct regions. For example, chimeric oligonucleotides may contain at least one region of modified nucleotides that confer beneficial properties (e.g. increased nuclease resistance, increased uptake into cells), or two or more oligonucleotides of the invention may be joined to form a chimeric oligonucleotide.

The aptamers of the present invention may be ribonucleic or deoxyribonucleic acids and may contain naturally occurring bases including adenine, guanine, cytosine, thymidine and uracil. The oligonucleotides may also contain modified bases such as xanthine, hypoxanthine, 2-aminoadenine, 6-methyl, 2-propyl and other alkyl adenines, 5-halo uracil, 5-halo cytosine, 6-aza uracil, 6-aza cytosine and 6-aza thymine, pseudo uracil, 4-thiouracil, 8-halo adenine, 8-aminoadenine, 8-thiol adenine, 8-thiolalkyl adenines, 8-hydroxyl adenine and other 8-substituted adenines, 8-halo guanines, 8-amino guanine, 8-thiol guanine, 8-thiolalkyl guanines, 8-hydroxyl guanine and other 8-substituted guanines, other aza and deaza uracils, thymidines, cytosines, adenines, or guanines, 5-trifluoromethyl uracil, and 5-trifluoro cytosine.

Other aptamers of the invention may contain modified phosphorous, oxygen heteroatoms in the phosphate backbone, short chain alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. For example, the aptamers may contain phosphorothioates, phosphotriesters, methyl phosphonates, and phosphorodithioates. In an embodiment of the invention there are

phosphorothioate bonds links between the four to six 3'-terminus bases. In another embodiment phosphorothioate bonds link all the nucleotides.

The aptamers of the invention may also comprise nucleotide analogs that may be better suited as therapeutic or experimental reagents. An example of an oligonucleotide analogue is a peptide nucleic acid (PNA) wherein the deoxyribose (or ribose) phosphate backbone in the DNA (or RNA), is replaced with a polyamide backbone which is similar to that found in peptides (P.E. Nielsen, et al Science 1991, 254, 1497). PNA analogues have been shown to be resistant to degradation by enzymes and to have extended lives *in vivo* and *in vitro*. PNAs also bind stronger to a complimentary DNA sequence due to the lack of charge repulsion between the PNA strand and the DNA strand. Other oligonucleotides may contain nucleotides containing polymer backbones, cyclic backbones, or acyclic backbones. For example, the nucleotides may have morpholino backbone structures (U.S. Patent No. 5,034,506). Oligonucleotides may also contain groups such as reporter groups, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an aptamer. Aptamers may also have sugar mimetics.

The aptamers may be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. The aptamers of the invention or a fragment thereof, may be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the protein-DNA interaction (e.g. phosphorothioate derivatives and acridine substituted nucleotides). The aptamer oligonucleotide sequences may be produced biologically using an expression vector introduced into cells in the form of a recombinant plasmid, phagemid or attenuated virus in which aptamer sequences are produced under the control of a high efficiency regulatory region, the activity of which may be determined by the cell type into which the vector is introduced.

Aptamers of the present invention also include any oligonucleotide having one or more additions and/or deletions relative to the sequence of an oligonucleotide whose sequence is shown herein, so long as the requisite GalNac or Tn antigen binding activity is maintained or increased. Requisite

binding activity is optionally determined by identifying whether binding occurs between the aptamer of the invention and a cell in the sample (for example, see below under the headings "Aptamer selection procedure for immobilized N-acetylgalactosamine" and "Electrophoretic Mobility Shift Assays (EMSA)." In one embodiment, a useful oligonucleotide is identified when the oligonucleotide complexes with MUC1 peptide and causes upward shift in the oligonucleotide electrophoretic mobility in a DNA retardation gel, such as a 6% polyacrylamide pre-cast DNA retardation gel. Threshold values for a selected aptamer would have its binding capacity from low picomolar affinity to and including 1 microMolar. A person skilled in the art will appreciate that other methods can be used to identify useful variants including flow cytometry, two-photon confocal microscopy, and BIAcore.

Aptamers of the present invention also include variants having at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 84%, at least 85%, at least 90%, at least 95% or at least 99% sequence identity to the sequences of the present invention, so long as the requisite GalNac or Tn antigen binding activity is maintained or increased. Sequence identity is most preferably assessed by the algorithms of BLAST (References to BLAST Searches include: Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990) "Basic local alignment search tool." J. Mol. Biol. 215:403.sub.-410; Madden, T. L., Tatusov, R. L. & Zhang, J. (1996) "Applications of network BLAST server" Meth. Enzymol. 266:131.sub.-141; Zhang, J. & Madden, T. L. (1997) "PowerBLAST: A new network BLAST application for interactive or automated sequence analysis and annotation." Genome Res. 7:649.sub.-656).

The term “fragment” or "part of the sequence" refers to any subject aptamer having an oligonucleotide sequence shorter than that of an oligonucleotide whose sequence is shown herein. Optionally one uses fragments comprising 7-9, 10-15, 16-20 or 21-24 nucleotides of the sequences provided in this application.

2. Uses of the Aptamers

The present invention includes all uses of the aptamers that bind to GalNac or the Tn antigen on the surface of cancer cells as described herein. The uses include the diagnosis, prognosis and treatment of cancer.

(a) Diagnosis or Monitoring of Cancer

In one embodiment, the present invention provides a use of an aptamer of the invention to diagnose or detect a cancer that is associated with the Tn antigen. This refers to a method or process of determining if an animal has or a sample contains cancerous cells that are associated with the Tn antigen.

The term "a cancer that is associated with the Tn antigen" means any type of cancer wherein the cancer or tumour cells have underglycosylated carbohydrate structures on their surface and/or they secrete or shed underglycosylated proteins, such as mucin glycoproteins, from their surface compared to a healthy wild type cell. Such cancers include breast, lung, colorectal, gastric, ovarian, colon, prostate, liver and pancreatic carcinomas. In one embodiment, the cancer includes breast or pancreatic carcinomas.

In another embodiment, the present invention provides a use of an aptamer of the invention as a prognostic tool or to monitor the efficacy of treatment of a cancer that is associated with the Tn antigen.

The present invention also provides a method of diagnosing or monitoring a cancer that is associated with the Tn antigen comprising contacting a sample from a patient with an aptamer of the invention and assaying for binding between the aptamer and a cancer cell in the sample, if present, wherein binding indicates the presence of a cancer that is associated with Tn antigen. Samples which may be tested include blood, urine, serum, tears, saliva, feces, tissues and the like. For solid tumors a tissue biopsy may be used.

The method used to detect the cancer can be any method known in the art. For example, the binding of the aptamer with the cancer cell or with a protein expressed on the surface of the cancer cell may be detected in known immunoassays which rely on the binding interaction between an aptamer of the invention and the cell or protein. Examples of such assays are

radioimmunoassays, enzyme immunoassays (e.g. ELISA), immunofluorescence, immunoprecipitation, latex agglutination, hemagglutination, and histochemical tests. The resulting cell or protein bound to the aptamer may be isolated by conventional isolation techniques, for example, salting out, chromatography, electrophoresis, gel filtration, fractionation, absorption, polyacrylamide gel electrophoresis, agglutination, or combinations thereof. Thus, the aptamers may be used to identify or quantify the amount of cancer cells or underglycosylated protein in a sample in order to diagnose the presence of a tumor.

For use in diagnostic assays, the aptamer is optionally labelled with a detectable marker including an enzyme, fluorescent compounds (eg. probes), luminescent compounds and radioactive compounds. Examples of suitable enzymes include horseradish peroxidase, biotin, alkaline phosphatase, ß-galactosidase, or acetylcholinesterase; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent compound includes luminol; and examples of suitable radioactive compounds include P-32, S-35, Cu-64, Ga-67, Zr-89, Ru-97, Tc-99m, Rh-105, Pd-109, In-111, I-123, I-125, I131, Re-186, Au-198, Au-199, Pb-203, At-211, Pb-212 and Bi-212. The aptamers may also be labelled or conjugated to one partner of a ligand binding pair. Representative examples include avidin-biotin and riboflavin-riboflavin binding protein. Methods for conjugating or labelling the ligands discussed above with the representative labels set forth above may be readily accomplished using conventional techniques.

In one embodiment, the aptamers of the invention are conjugated to a detectable marker to detect the presence of a cancer cell or underglycosylated protein in a sample. In another embodiment, the aptamers are not conjugated to a detectable marker in order to detect the presence of a cancer cell or underglycosylated protein in a sample. For example, diffractive optics technology (e.g. dot^{TM}, Alexla Biosensors) can be used to detect binding of the aptamer, whether or not the aptamer is conjugated to a detectable marker.

The aptamers of the invention are useful in immuno-histochemical analyses, for example, at the cellular and sub-subcellular level, to detect cancer cells or underglycosylated proteins, to localise it to particular cells and tissues, and to specific subcellular locations, and to quantitate the level of expression. The aptamers of the invention may also be used in flow cytometry.

Cytochemical techniques known in the art for localizing DNA using light and electron microscopy are useful to detect the cancer cell or protein. Generally, an aptamer of the invention is readily labelled with a detectable substance and the cancer cell or protein is localised in tissues and cells based upon the presence of the detectable substance. Examples of detectable substances include various enzymes such as biotin, alkaline phosphatase, ß-galactosidase, or acetylcholinesterase; fluorescent materials such as fluorescein; luminescent materials such as luminol; and, radioactive materials such as radioactive iodine I^{125}, I^{131} or tritium. Ligands may also be coupled to electron dense substances, such as ferritin or colloidal gold, which are readily visualised by electron microscopy.

The aptamer may be immobilized on a carrier or solid support such as nitrocellulose, glass, polyacrylamides, gabbros, and magnetite. The support material may have any possible configuration including spherical (e.g. bead), cylindrical (e.g. inside surface of a test tube or well, or the external surface of a rod), or flat (e.g. sheet, test strip). When an insolubilized ligand is used protein bound to the ligand is isolated by washing. For example, when the sample is blotted onto a nitrocellulose membrane, the ligand bound to the cancer cell or protein is separated from the unreacted ligand by washing with a buffer, for example, phosphate buffered saline (PBS) with bovine serum albumin (BSA).

When labelled aptamer is used, the presence of a tumor can be determined by measuring the amount of labelled aptamer bound to the cancer cell or protein in the sample or of the unreacted labelled aptamer. The appropriate method of measuring the labelled material is dependent upon the labelling agent.

Where a radioactive label is used as a detectable substance, the cancer cell or protein may be localized by radioautography. The results of

radioautography may be quantitated by determining the density of particles in the radioautographs by various optical methods, or by counting the grains.

When unlabelled aptamer is used in the method of the invention, the presence of the cancer cell or protein can be determined by measuring the amount of aptamer bound using substances that interact specifically with the aptamer to cause agglutination or precipitation. In particular, labelled antibody against a ligand of the invention, can be added to the reaction mixture. The presence of the cancer cell or protein can be determined by a suitable method from among the already described techniques depending on the type of labelling agent.

The reagents suitable for carrying out the diagnostic methods of the invention are optionally packaged into kits providing the necessary materials, packaged into suitable containers. Such kits optionally include all the reagents required to detect a cancer cell or protein having a Tn antigen on its surface in a sample by means of the methods described herein, and optionally suitable supports useful in performing the methods of the invention. The kit comprises at least one aptamer of the invention and instructions for the use thereof, for example, in diagnosing cancer by detecting if the Tn antigen is present in the sample by determining whether binding occurs between the aptamer and any cell or protein in the sample, wherein binding indicates the presence of a cancer that is associated with the Tn antigen.

(b) Therapeutic Uses

In a further embodiment, the present invention provides a use of an aptamer that binds to GalNac or the Tn antigen to treat or prevent a cancer that has increased underglycosylated sugar levels. In such an embodiment the aptamer may be coupled to an anticancer agent and used to target the agent to the cancer. The aptamer is optionally coupled to a drug, a radionuclide, a toxin, a cytotoxic peptide, an oligonucleotide or a plasmid comprising a gene encoding a therapeutic agent. In one embodiment, the aptamer is conjugated to Chlorin e6 (Ce6).

The phrase "to treat or prevent cancer" as used herein includes inhibiting the disease, preventing the disease or reducing the symptoms associated with the disease.

The cancer may be selected from the group consisting of breast, lung, colorectal, gastric, ovarian, colon, prostate, liver and pancreatic carcinomas. In one embodiment, the cancer is breast or pancreatic cancer.

Accordingly, the present invention provides a use of an aptamer of the invention to prepare a medicament to treat or prevent a cancer that is associated with the Tn antigen. The present invention also provides a use of the aptamer of the invention to treat or prevent a cancer that is associated with the Tn antigen. The invention further includes a method of treating or preventing a cancer associated with the Tn antigen comprising administering an effective amount of the aptamer of the invention coupled to an anticancer agent to an animal in need thereof.

The term “animal” as used herein includes all members of the animal kingdom. Preferably, the animal is a mammal, more preferably a human.

The aptamers coupled to anticancer agents are readily formulated into pharmaceutical compositions for administration to subjects in a biologically compatible form suitable for administration in vivo. By "biologically compatible form suitable for administration in vivo" is meant a form of the substance to be administered in which any toxic effects are outweighed by the therapeutic effects. The substances may be administered to living organisms including humans, and animals. Administration of an "effective amount" of the pharmaceutical compositions of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. For example, a therapeutically active amount of a substance may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of antibody to elicit a desired response in the individual. Dosage regima is typically adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

The active substance may be administered in a convenient manner such as by injection (subcutaneous, intravenous, etc.), oral administration, inhalation, transdermal application, or rectal administration. Depending on the route of administration, the active substance may be coated in a material to

protect the compound from the action of enzymes, acids and other natural conditions which may inactivate the compound.

The compositions described herein can be prepared by per se known methods for the preparation of pharmaceutically acceptable compositions which can be administered to subjects, such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example, in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA 1985). On this basis, the compositions include, albeit not exclusively, solutions of the substances in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and iso-osmotic with the physiological fluids.

The invention also provides a method of profiling a cancer cell type, comprising i) contacting a sample from a subject with the aptamer of the invention; ii) detecting binding between the cell in the sample and the aptamer, and iii) determining the amount of aptamer bound to the cell, thereby determining the type of cancer cell present in the sample.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples. These examples are described solely for the purpose of illustration and are not intended to limit the scope of the invention. Changes in form and substitution of equivalents are contemplated as circumstances might suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitation.

The following non-limiting examples are illustrative of the present invention:

EXAMPLES

Example 1:

Sequences obtained:

1 AGACTTAGGTGGATGTAGGATCCTT (SEQ ID NO:3)

2 CTCCGATCCACTAGTAACGGCCGCC (SEQ ID NO:7)

3 AAGGGATGACAGGATACGCCAAGCT (SEQ ID NO:14)

4 AACGCAGTACAGGATACGCCAAGCT (SEQ ID NO:15)

Sequence 3 and 4 belong to the same family (highly conserved).

Methodology

SELEX Library used :

5' GGGAGACAAGAATAAACGCTCAA (N25) TTCGACAGGAGGCTCA CAACAGGC '3 (SEQ ID NO:19)

SELEX Methodology

Aptamers recognizing GalNac (Tn antigen) were identified using the SELEX (Systematic Evolution of Ligands by EXponential enrichment) methodology (Ellington, 1990 and Tuerk, 1990) in the context of a 96-well plate format assay. Briefly, 96-well strip plates were purchased to VWR (EMD biosciences, Calbiochem®, Mississauga, On, Ca). GalNac is covalently coupled to the polystyrene matrix of plastic wells using an amide linkage harbouring a 16 - 18 atom flexible spacer (Figure 1). This linker thus provides a homogeneous presentation of the target sugar to potential SELEX binding candidates. Panning stages (binding and elution steps) were performed in physiological buffers in order to identify specific ssDNA aptamers folded under such native conditions from the selection methodology. The binding and wash buffers were composed of 100mM NaCl, 5mM $MgCl_2$, pH7.4, while the elution buffer was of higher ionic strength, i.e. 1.5M NaCl, 5mM $MgCl_2$, pH7.4. An aptamer library composed of two non-randomized flanking regions and a random 25-base central region was panned for binding to the sugar in a plate well. The incubation step lasted 1 hour at 37^0C. The well was then washed 3 times with wash buffer and bound aptamers were eluted twice with elution buffer. The recovered pool of aptamers was subsequently desalted and amplified by standard PCR for 35 rounds. This enriched pool of ssDNA constituted a round of selection. The panning cycle was performed 10 times.

After 10 rounds of selection, the final pool of aptamers was cloned into a pCR ®2.1Topo vector (Invitrogen), and clones were analysed and sequenced to reveal aptamers sequences showing specificity for the GalNAc sugar. Sequencing was performed by Macrogen (Seoul, Korea). Sequences were aligned and analysed by BioEdit sequence alignment editor (Tom Hall, Ibis therapeutics, Carlsbad, Ca, USA). 90% of the candidate aptamers identified in the last pool of aptamers were represented by the sequences disclosed herein.

Example 2:

MATERIALS AND METHODS

DNA library and primers

The DNA oligonucleotide library contained a 25 base central random sequence flanked by primer sites on either side: (5′-GGGAGACAAGAATAAACGCTCAA-N25-TTCGACAGGAGGCTCACAACAGGC-3′) (Fitzwater and Polisky 1996). The forward primer (5′-GGGAGACAAGAATAAACGCTCAA-3′) (SEQ ID NO: 20) and reverse primer (5′-GCCTGTTGTGAGCCTCCTGTCGAA-3′) (SEQ ID NO: 21) were used in asymmetric PCR to generate single-stranded DNA elements (Missailidis *et al.* 2005). The library and all primers were synthesized at the Oligonucleotide and Peptide Synthesis Unit of the Queen's Medical Centre, University of Nottingham (Nottingham, UK).

Preparation of unglycosylated and Tn-labelled mucin MUC1-5TR

The preparation and glycosylation of a 5-tandem repeat mucin MUC1 peptide, termed MUC1-5TR, was previously described (Price *et al.* 1998). Briefly, a synthetic gene coding for five copies of the MUC1 tandem repeat and a terminal hexahistidine metal affinity tag (MUC1-5TR) was inserted into the pET-15b expression vector (Novagen, Madison, WI). The resulting construct was used to transform competent *E. coli* BL21 (DE3) cells (Novagen) and the expression of MUC1-5TR induced with isopropyl β-D-thiogalactopyranoside (IPTG). MUC1-5TR was purified by nickel affinity chromatography under denaturing conditions. The sequence of the purified

MUC1-5TR was confirmed by mass spectrometry and the peptide was stored at -20^{0}C until use. MUC1-5TR was enzymatically modified with up to 15 GalNac sugars (Tn antigen) using a recombinant secreted form of the human ppGalNAc-T1 expressed in *Pichia pastoris* (Brokx *et al.* 2003).

Preparation of immobilized MUC1-5TR peptide affinity columns

One-millilitre HiTrap Chelating HP columns were purchased from Pharmacia (Uppsala, Sweden). The columns were functionalised with nickel (Ni^{2+}) according to the manufacturer's instructions. Unglycosylated MUC1-5TR or its Tn-labelled form (3 mg) were separately immobilized unto the column matrix and subsequently used for aptamer selection.

Preparation of N-acetylgalactosamine target

Plates covalently modified with *N*-acetylgalactosamine were purchased from EMD biosciences (Mississauga, ON, Ca). Briefly, GalNac determinants were directly coupled using an amide linkage to wells of a 96-well polystyrene plate via a 16 - 18 atom flexible spacer, providing a homogeneous presentation of the target to putative DNA aptamers emerging as strong ligands. Plates were kept at -20^{0}C for the duration of the experiment, except during ssDNA library incubation times.

Aptamer selection procedure using peptide targets immobilized on nickel affinity columns

DNA aptamers with a high affinity to unglycosylated and Tn-labelled mucin MUC1-5TR were selected as follows. A single stranded DNA pool was generated from the original synthetic pool by large-scale unidirectional PCR in order to enrich for the presence of all DNA aptamer species in the original library. Newly amplified ssDNA pools were denatured by heating at 94°C for 10 min in a selection buffer containing 100 mM NaCl, 5 mM $MgCl_2$ (pH 7.4) and then renatured at room temperature. The amplified ssDNA library was loaded and incubated onto a HiTrap nickel column saturated with either MUC1-5TR or MUC1-5TR-GalNac peptided serving as a target. DNA-peptide complexes were left to form at 37°C for 60 min in the selection buffer. Partitioning of bound and unbound ssDNA sequences was performed in two

steps. The column was first washed with 5 column volumes of 150 mM NaCl, 5 mM $MgCl_2$, pH 7.4 and the remaining bound aptamers were eluted with 3 column volumes of a high-molarity NaCl solution (1500 mM NaCl, 5 mM $MgCl_2$, pH 7.4). The eluted DNA pool was desalted using a NAP-25 column (GE Healthcare) and lyophilized overnight. Binding aptamers were amplified by PCR. Forward and Reverse primers were used for asymmetric PCR amplification (35 cycles of 1 min at 94°C, 1 min at 56°C, and 1 min at 72°C, followed by 10 min at 72°C). The resulting pooled ssDNA served as the enriched library for the next selection round. Ten rounds of selection were performed prior to analysing aptamer candidates populating the final ssDNA pool.

Aptamer selection procedure for immobilized N-acetylgalactosamine

The procedure for identifying DNA aptamers with a high affinity to N-acetylgalactosamine followed the identical strategy described for the immobilized MUC1-5TR peptides with the following exceptions. The amplified ssDNA library was dispensed in wells derivatized with GalNac sugars. The library was incubated at 37°C for 60 min in the selection buffer. The wells were then washed with two 100µL volumes of 150 mM NaCl, 5 mM $MgCl_2$, pH 7.4. The bound aptamers were then released with two 100µL volumes of a high-molarity NaCl solution (1500 mM NaCl, 5 mM $MgCl_2$, pH 7.4). The eluted DNA pool was recovered and a similar iterative selection strategy (10 cycles) of aptamer pool eventually led to the identification of aptamers specific for N-acetylgalactosamine.

Cloning and sequencing

DNA aptamers obtained from the tenth round of selection were amplified by PCR using unmodified primers and cloned into *Escherichia coli* using the TA cloning kit (Invitrogen, Burlington, Ontario, Canada). Plasmids from individual clones were isolated and sequenced.

Electrophoretic Mobility Shift Assays (EMSA)

The association of DNA aptamers recognizing MUC1 peptide constructs with their respective target was confirmed by a shift in their electrophoretic mobility in a 6% polyacrylamide pre-cast DNA retardation gel (Invitrogen, Burlington, Canada) upon complexation with their target. Qualitative EMSA was performed using a binding reaction mixture (20 µl) composed of 100 mM NaCl, 5 mM MgCl, 100 mM KCl, 1mM dithiothreitol, 5% glycerol, a protein target (5µL, 1mg/mL) and a ^{32}P-labeled DNA aptamer. The mixture was incubated for 30 min at room temperature and electrophoresed at a constant voltage of 150 V for 2 h at room temperature. The running buffer was 1/2 × TBE (0.045 M Tris borate, pH 8, 1 mM EDTA). The gel was subsequently dried and exposed to film.

Surface plasmon resonance (SPR) measurements

All SPR binding measurements were recorded on a BIAcore 3000 instrument using nickel NTA sensor chips (CM5 chip; Uppsala, Sweden). The MUC1 peptide antigens carry a hexa histidine tag and were directly immobilized onto NTA chips by dissolving each antigen in 10 mM sodium acetate (pH 5.2) and injecting them onto a chip at a concentration of 500µg/ml. The chips were equilibrated in a binding buffer (100 mM NaCl, 5 mM $MgCl_2$ (pH 7.4) that also served as the running buffer. The flow rate was 5 µL/min. The initial association phase of the sensograms was fitted by non-linear regression analysis to a one-phase exponential association model expressed by the following equation: **[Response]=[Plateau]+[Span](1−exp (−*k*(*t*−t_0)))**.

Where k is the apparent concentration-dependent association rate constant.

Similarly, the dissociation phase of the sensograms was subjected to a non-linear regression analysis based on a one-phase exponential association model using the expression: **[Response]=[Bottom]+([Plateau]−[Bottom]) exp (−k_d(*t*−t_0))**.

The concentration of free aptamer was assumed to be constant since an excess amount of aptamer was applied to the system in relation to the amount of target peptide. Therefore the term k equals k_aC+k_d, where C

represents the total aptamer concentration in the system and the small letter k_a corresponds to the intrinsic association rate constant. Therefore k_a= (K-k_d)/C and the value of K_d equals k_d/k_a. Statistical analyses were performed using the software package Origin version 6.0 (Microcal, Massachusetts, USA), with χ^2 values reflecting the goodness of fit of the data to the model.

The DNA aptamers were refolded in the binding buffer (100 mM NaCl, 5 mM $MgCl_2$, pH 7.4) for the dose-dependent binding assays and injected over the flow cells. The surfaces were regenerated between injections using two 5-µL injections of 10 mM NaOH, followed by a 10-minute wash step with the running buffer. The association and dissociation rate constants for aptamer-peptide and aptamer-sugar complexes were determined using a non-linear regression analysis for the initial parts of association and dissociation phases of the sensograms (7.5 Origin Pro software; MicroCal).

Flow cytometry

Rhodamine-labeled aptamers (200 pmol) were incubated with 10^5 cells of either MCF-7, T47D, PANC-1 or CHO cells at 37°C for 30 min to determine the level of MUC1 expression on their surface. Cells were trypsinized, washed three times with PBS and counted using a hemocytometer. The cells were resuspended in 500µl of PBS and 4% FBS and incubated with either 200pmol of a rhodamine-labelled aptamer or with the mAb *Onc*M27(44) for 30 min at 37^0C. Cells were subsequently washed three times with PBS and the antibody-stained cells were further incubated with 1:1500 dilution of FITC-labelled anti-mouse antibody (Pharmingen) in PBS. After a 30-minute incubation period, cells were washed three times with PBS and analysed by flow cytometry (FACScan; BD Biosciences, Franklin Lakes, NJ).

FACS Internalization studies

Flow cytometry studies were carried out as above, with the added modifications. All aptamer and mAb *Onc*M27 were incubated at 37^0C in the incubator or at 4^0C on ice for the period of 30 min, 1hour, 2hours, 4 hours, 6 hours. FACS measurements and analysis were performed at each time point.

Confocal microscopy

MCF-7, T47D, PANC-1 or CHO cells were grown in duplicate wells of 4-well chambered coverglass tissue culture microscopy plates (NUNC). Cell medium was removed from each well and cells were further washed 3 times with PBS. Rhodamine-labelled aptamers or the mAb OncM27 were dissolved in PBS and added to the wells at concentrations ranging from 0.05 pmol/μL to 1 pmol/μL. Plates were incubated for 30 min at 37^0 C. Wells were washed three times with PBS and treated with 2μL per 1000μL of a 2% (w/v) BSA solution prepared in PBS. In the case of the mAb OncM27, a second labelling step was necessary with a FITC-labelled anti-mouse antibody (Pharmingen, Becton Dickinson) for 30 min at 37^0 C. Cells were washed three times and kept in 20mM Hepes buffer prior to microscopy. Either 100 μL of PBS (control) or 100 μL of a 0.25 pmol/μL solution of a fluorescently-labelled aptamer in PBS were dispensed in each well. The DNA aptamers were allowed to complex with MUC1 targets present on the surface of cells for 30 minutes. Individual wells were washed three times with 1 millilitre of PBS. Adherent cells in wells were visualized using a Two-Photon Zeiss LSM 510 META NLO microscope, HeNe Laser - (543nm) HeNe Laser - (633nm), C-Apo 40x/1.2 NA lens, at 40x1.6x10 magnification, and analyzed with LSM150 image browser software. Phase-contrast and confocal images (2-micron thickness) of viable cells were taken after the 30-minute incubation period.

Chlorin E6 (Ce6) treatment

Aliquots of $5x10^3$ cells of either T47D or CHO cells suspended in 200μL growth medium were seeded into wells of blackened 96 well plates with clear bottoms (Cornig Costar Corp. , Cambridge, MA) and cultured for 6h at 37^0C in the presence of 5% CO_2. Cells as triplicate cultures were treated with PBS and increasing concentrations of either Chlorin E 6 (0.2-100mM) or Chlorin E 6 conjugated aptamer (0.2nM-100nM) followed by dark (control) or light activation treatments. Wells were irradiated with 664nm light from the above using an optic fiber coupled to a dye laser source (Spectra Physics model 375B), applying a radiant exposure of 12 J/cm^2 at a fluence rate of 20-30 mW/cm^2. Once irradiated, cells were incubated with fresh growth medium for an additional 48 h at 37 °C in the dark. The photoinduced thermal effect

was measured in individual wells an in vitro MTT toxicity assay kit (Sigma Aldrich).

Results

The relevant and unique tumor antigens associated with the mucin MUC1 tandem repeat are composed of both peptide and carbohydrate determinants. The invention satisfies the strong need for low molecular weight probes able to precisely recognize molecular features distinguishing such markers from glycosylated MUC1 forms present on normal epithelial cells. The invention provides isolated, short oligonucleotide aptamers tailored to specifically recognize underglycosylated forms of the mucin MUC1 tandem repeat.

Aptamer selection and characterization- Mimics of known mucin MUC1 determinants on cancer cells, namely a recombinant deglycosylated peptide representing 5 MUC1 tandem repeats (MUC1-5TR) and its *O*-glycosylated form containing 15 GalNac sugars (Tn antigens; MUC1-5TR-GalNac), were initially prepared as previously described (Brokx *et al.* 2003) to later serve as targets in selecting useful DNA aptamers. In addition, an immobilized form of N-acetylgalactosamine (GalNac) was also used as a target to refine the selectivity of the identified family of MUC1-directed DNA aptamers. Two *in vitro* selection strategies based on the SELEX approach (Ellington and Szostak 1990, Tuerk and Gold 1990, Missailidis *et al.* 2005) were developed to identify aptamers that would bind to their targets under physiological conditions (37^0C, pH 7.4) (Fig 2). In the case of MUC1-5TR and MUC1-5TR-GalNac (Tn antigen), the peptides were immobilized onto nickel chelating resin beads by virtue of their histidine tag while the carbohydrate GalNac was covalently linked to wells of a 96-well plate. A synthetic library composed of 4^{25} random sequence single stranded DNA molecules was initially amplified to generate a higher copy number and both protein (MUC1-5TR and MUC1-5TR-GalNac) and carbohydrate (GalNac alone) targets were subsequently exposed to the resulting pool of random ssDNA molecules. Aptamers displaying strong binding were released from their immobilized target in high salt (1.5M NaCl) and amplified by asymmetric PCR techniques (Daniels *et al.*

2003, Mori *et al.* 2004). The products of PCR amplification were then used as templates for the next *in vitro* selection round. Ten rounds of iterative selection and amplification were performed and at least 50 randomly picked plasmid clones following the final round of each target selection were sequenced (Macrogen, Korea). Ninety percent of all sequences observed were represented by four aptamers binding to MUC1-5TR, by four aptamers recognizing the MUC1-5TR-GalNac construct, and by three *N*-acetylgalactosamine binding aptamers. These oligonucleotide sequences are listed in Table 1. The aptamer sequences obtained in the screening against GalNac include 3 families of sequences as listed below.

Family 1:

AGACTTAGGTGGATGTAGGATCCTT (SEQ ID NO:3)
GAACTTAGGTGGATGTAGGATCCTT (SEQ ID NO:4)
AGACTTAGGTGGATGTAGGATCCAA (SEQ ID NO:5)
GGACTTAGGTGGATGTAGGATCCTT (SEQ ID NO:6)

Family 2:

CTCCGATCCACTAGTAACGGCCGCC (SEQ ID NO:7)
GACCGATCCACTAGTAACGGCCGCC (SEQ ID NO:8)
AGCCGATCCACTAGTAACGGCCGCC (SEQ ID NO:9)
AACCGATCCACTAGTAACGGCCGCC (SEQ ID NO:10)
TACCGATCCACTAGTAACGGCCGCC (SEQ ID NO:11)
GACCGATCCACTAGAAACGGCCGCC (SEQ ID NO:12)
GACCGTACCACTAGTAACGGCCGCC (SEQ ID NO:13)

Family 3:

AAGGGATGACAGGATACGCCAAGCT (SEQ ID NO:14)
AACGCAGTACAGGATACGCCAAGCT (SEQ ID NO:15)
AACCCATGACAGGATACGCCAAGCT (SEQ ID NO:16)
TGCGGATGACAGGATACGCCAAGCT (SEQ ID NO:17)
TAGGCTAGACAGGATACGCCAAGCT (SEQ ID NO:18)

Probing aptamer affinity and specificity- The ability of each aptamer to recognize its cognate target was evaluated using an electrophoretic mobility

shift assay (see materials and methods). All aptamers' bands demonstrated a characteristic upward shift in their mobility as expected upon complexation with their target (Fig 3). Interestingly, DNA aptamers derived to either glycosylated targets (Tn antigen and GalNac) displayed non-equivalent band migration shifts with their target. Glycosylated MUC1 (Tn antigen peptide) may harbor 10 to 15 GalNac groups, within the MUC1-5TR scaffold (Brokx *et al.* 2003), a feature that may explain the presence of more than one-shifted bands representing the most abundant species present in the MUC1-5TR-GalNac sample.

The affinity of aptamers for their target was defined by surface plasmon resonance (SPR) spectroscopy (BIAcore), a method validated in the past by other investigators (Fujino T *et al.* 2003). Both peptide targets were immobilized on the surface of nickel NTA sensor chips. This immobilization method mimics the approach used in our selection strategies. Association and dissociation constants were derived from SPR measurements (Table 2).

The K_d values (dissociation constant) found for aptamers to the 5TR MUC1 backbone varied between 47.3 nM and 85.0 nM, aptamers for the Tn Antigen varied between 18.6 and 25.1nM and aptamers for the *N*-acetylgalactosamine varied between 34.4 and 59.8. DNA aptamers evolved against the MUC1-5TR-GalNac (Tn antigen) displayed a tighter binding constant (lower K_d values) than aptamers recognizing the carbohydrate determinant GalNac alone. This feature has been observed in the past for antibodies where the presence of carbohydrate moieties allowed for a more stable interaction of the antibody with its target (Linsley et al. 1988). DNA aptamers derived to the *N*-acetylgalactosamine sugar alone, were found to typically have marginally lower affinities (4-5 fold). Nevertheless, K_d values in the nanomolar range for an aptamer binding to a carbohydrate determinant is comparable to binding affinities observed for much larger bivalent antibodies and multivalent lectins (Thomas *et al.* 1999).

Selected aptamers bind specifically to cells expressing MUC1 glycoforms- DNA aptamers displaying the highest affinity constant for their respective target, namely 5TR-1, 5TRG-2 and GalNac-3, were re-synthesized

with a 5 prime end-labeled Rhodamine tag to analyse their binding to cells expressing MUC1 glycoforms. Specifically, the binding of fluorescent DNA aptamers to three MUC1-expressing cell lines, MCF-7 (human breast cancer), T47D (human breast cancer) and Panc-1 (human pancreatic cancer) as well as the MUC1 negative control cell line CHO (Chinese hamster ovary), was analyzed by flow cytometry as well as by two-photon confocal microscopy. The monoclonal antibody *Onc*M27 (Litvinov 1993, Ceriani 1992, Altschuler 2000, Henderikx 2002) recognizes the peptide epitope TRP within the MUC1 tandem repeat and was used as a control to demonstrate the expression of MUC1 mucin on all cell lines tested.

The flow cytometric profiles presented in Fig.4, demonstrate that all three fluorescent aptamers can recognize their determinants on epithelial cancer cells known to express MUC1 epitopes while the mAb *Onc*M27 or the rhodamine-labeled aptamers fail to bind to CHO cells. Interestingly, the aptamers provided signals of comparable magnitude to the mAb *Onc*M27 and allow one to dissect the presence of both MUC1 peptide and carbohydrate determinants on cells. In particular, the fluorescence signals observed in the case of MCF-7 cells for the three aptamers show that this cell line exist as a heterogeneous population of cells differing in the levels of expression of these peptide/carbohydrate markers. This feature was not observed from the signal arising from using the mAb *Onc*M27 as a detection reagent.

The confocal images presented in Fig. 5 support the flow cytometry data and further illustrate that the chosen aptamers are able to recognize either the MUC1 peptide backbone or GalNac determinants associated or not with the mucin MUC1 tandem repeat. As expected, no cell surface staining was observed for MUC1-negative CHO cells. Furthermore, the visualized plane within cells (2-micron thickness planes) shows that aptamers are internalized by all three MUC1 positive cell lines with the greater extent being observed for T47D cells. The anionic character of oligonucleotides does not favor their spontaneous entry into cells. However, epithelial cells are known to typically endocytose and recycle underglycosylated forms of mucin MUC1 from their surface leading to further modifications of their glycan structure (Price *et al.* 1998, Spencer *et al.* 1999, Burton *et al.* 1999). The internalization

of mucin MUC1 is particularly observed in cancer cells lines displaying aberrant glycosylation patterns.

Internalization of selected aptamers that bind specifically to cells expressing MUC1 glycoforms- To further demonstrate the method of internalization, and to show that binding to the membrane associated tumour marker is responsible receptor-mediated endocytosis, aptamers were incubated with the MUC-1 expressing tumour cell lines and the CHO control cell line. The study was performed at the biologically active temperature of 37^0C for 6 hours, and compared to internalization at 4^0C, where little to none receptor-mediated endocytosis occurs, and also in the presence of a known receptor-mediated endocytosis inhibitor monodansyl cadaverine. The flow cytometry profiles were ploted and interesting patterns of internalization and recognition were observed. As expected, all aptamers were successfully internalized at 37^0C. In general the plateau of internalization was reached at the two hour time point. All aptamers showed reduced internalization in the presence of monodansyl cadaverine. The control aptamer (scrambled aptamer) was not able to recognize any of the cell lines. In particular the Tn antigen aptamer showed higher levels of recognition in the cell lines T47D and PANC-1. Both cell lines have been documented in literature to have a higher number of this molecule when compared to cell lines like MCF-7.

Cell killing mediated by selected aptamers targeted specifically to cells expressing MUC1 glycoforms- Additionally, to demonstrate ability of these aptamers as therapeutics, the aptamers were synthesized with a conjugated Ce6 molecule for photodynamic therapy. These aptamers selectively target MUC-1 presenting cancer cells and enhance photodynamic cell killing when compared to free Ce6. Direct comparisons of dark versus light toxicities of all compounds conjugated to Ce6 or not, or free Ce6 was derived in titration studies. Potentiation of the aptamer conjugated Ce6 was demonstrated to be more efficient than delivery of free Ce6. This enhancement is due to the accumulation and localization of the photosensitizer intracellularly when compared to free compound.

Discussion

Specificity is a crucial parameter in devising targeted cancer therapies that can home on cancer cells while sparing normal tissues. However, many of the best-known tumour markers are termed tumour-associated antigens, meaning that such antigens are overexpressed on malignant cells and to a lesser extent on normal cells. In the case of aberrantly glycosylated mucin MUC1 antigens, the complex nature of the resulting peptide-carbohydrate determinants provides molecular signatures that are simply not observed on normal cells. This type of unique tumour antigen is utilized in the present invention which targets by the mucin MUC1 peptide tandem repeat harbouring *O*-linked Tn antigens. The invention allows precise recognition of both peptide and carbohydrate moieties within this tumour antigen uses ligands that can span a large surface area while coping with the inherent conformational flexibility of such determinants. The present invention provides three classes of DNA aptamers (Figs. 2 and 3) that recognize either the mucin MUC1 peptide repeat (MUC1-5TR), the MUC1 peptide repeat harbouring up to 15 GalNac groups (Tn antigen) or the carbohydrate N-acetylgalactosamine (GalNac). All DNA aptamers (Table 1) displayed binding affinities in the nanomolar range (Table 2) and selectivity for their given targets (Fig. 3-5). In terms of specificity, rhodamine-labeled aptamers were shown to recognize their respective targets on the breast cancer cell lines MCF-7, T47D as well as on the pancreatic cancer cell line PANC-1, but not on a MUC-1 negative cell line (CHO) (Fig. 4 and 5). These aptamers were also able to resolve the combined expression of complex peptide-carbohydrate tumour markers as in the case of the breast cancer cell line MCF-7 (Fig. 4). Specifically, the heterogeneity of expression of the Tn antigen and GalNac moieties on MCF-7 cells could be directly visualized as a staining pattern displaying more than one observable cell population. This feature was not resolved with the use of the mAb *Onc*M27 or the aptamer directed only at the MUC1 peptide tandem repeat. Finally, the cellular targeting as well as the internalisation of rhodamine-labelled aptamers by cancer cells was observed within a half-hour of their exposure to such ligands, with internalization peak being reached after two hours (Fig.5 and Fig.6). Furthermore, analysis of route of internalization was performed by comparison of rates of internalization a 37^0C and 4^0C and

the use of an inhibitor of receptor-mediated internalization. These studies demonstrated that this route is heavily used for the routing of these molecules to the intracellular domain. The same method of internalization was shown in the past for an RNA aptamer that recognizes the tumour marker PSMA. The N-acetylgalactosamine aptamer showed higher levels of recognition for all cell lines when compared to the other aptamers. This indicates the recognition of other sugar molecules present at the cell surface. This aptamer thus shows a wide web of applicability than the aptamers directed at the Tn antigen or the MUC1 core alone as a diagnostic or therapeutic. These findings, thus show that these oligonucleotides are useful delivery vehicles (Litvinov and Hilkens 1993, Ceriani et al. 1992, Altschuler et al. 2000, Henderikx et al. 2002). Specifically, aberrant *O*-glycan structures on mucin MUC1 stimulates its endocytosis and intracellular accumulation allowing the transit of aptamers as far as the trans-Golgi compartment (TGN) (Fig.6) where mucin *O*-glycosylation events can take place (Li *et al.* 2003). Interestingly, other organelles are readily targeted using this aptamer-MUC1 routing feature since MUC1 has also been observed in the past to traffic to other cellular compartments (nucleus and mitochondria) in breast, pancreatic and colon carcinoma cell lines (Tuerk and Gold 1990, Li *et al.* 2003, Wen *et al.* 2003, Ren *et al.* 2004).

In summary, the invention provides derived DNA aptamers directed at mucin MUC1 determinants to take advantage of the cellular feature that this membrane glycoprotein is highly expressed and aberrantly glycosylated in greater than 90% of all primary and metastatic breast cancers (Fitzwater and Polisky 1996, Missailidis *et al.* 2005, Brokx *et al.* 2003) as well as most epithelial carcinomas (Gendler 2001). Targeting small DNA aptamers to the underglycosylated determinants of MUC1 thus is useful to readily determines the status on epithelial cancer cells and to direct therapeutic agents to and into such cells.

While the present invention has been described with reference to what are presently considered to be the preferred examples, it is to be understood that the invention is not limited to the disclosed examples. To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety

Table 1

	5'> GGGAGACAAGAATAAACGCTCAA <3' (Forward primer) 5'>GCCTGTTGTGAGCCTCCTGTCGAA<3' (Reverse primer) 5' 5'GAGACAAGAATAAACGCTCAA-(N25)-TTCGACAGGAGGCTCACAACAGGC '3		
5TR-1	GAAGTGAAAATGACAGAACACAACA	(SEQ ID NO: 22)	25nt
5TR-2	GGCTATAGCACATGGGTAAAACGAC	(SEQ ID NO: 23)	25nt
5TR-3	CAAACAATCAAACAGCAGTGGGGTG	(SEQ ID NO: 24)	25nt
5TR-4	TACTGCATGCACACCACTTCAACTA	(SEQ ID NO: 25)	25nt
5TRG-1	GGGTTATATTACTCGGCCGGTGTAA	(SEQ ID NO: 26)	25nt
5TRG-2	GGCTATAGCACATGGGTAAAACGAC	(SEQ ID NO: 27)	25nt
5TRG-3	GGCGTACGGTAGGCGGGGTCAACTG	(SEQ ID NO: 28)	25nt
5TRG-4	GCTGGGTAAATAGATGATTCCCGGC	(SEQ ID NO: 29)	25nt
GalNac-1	AGACTTAGGTGGATGTAGGATCCTT		25nt
GalNac-2	CTCCGATCCACTAGTAACGGCCGCC		25nt
GalNac-3	AAGGGATGACAGGATACGCCAAGCT		25nt

Table 1- List of 11 oligonucleotide sequences representing ssDNA aptamers most frequently observed within the pool of selected aptamers. The sequences between the unique priming sites are shown along with the nucleotide length. The synthetic aptamer library and primers used for the asymmetric PCR amplification steps are described at the top of the table.

Table 2

Aptamer	SELEX Target	k_a(M-1s-1)	k_d (s-1)	K_d(nM)	χ^2
5TR-1	MUC1-5TR	$1.22x10^7$	0.0026	21.0	±0.992
5TR-2	MUC1-5TR	$1.48x10^7$	0.01268	85.2	±0.986
5TR-3	MUC1-5TR	$0.43x10^7$	0.00358	83.0	±0.981
5TR-4	MUC1-5TR	$0.45x10^7$	0.00311	69.0	±0.974
5TRG-1	Tn Antigen	$1.33x10^7$	0.00398	25.1	±0.995
5TRG-2	Tn Antigen	$1.02x10^7$	0.0186	18.6	±0.994
5TRG-3	Tn Antigen	$0.92x10^7$	0.00252	27.2	±0.996
5TRG-4	Tn Antigen	$0.91x10^7$	0.00314	34.4	±0.996
GalNac-1	*N*-acetylgalactosamine	$1.33x10^7$	0.0801	59.8	±0.991
GalNac-2	*N*-acetylgalactosamine	$2.02x10^7$	0.1199	58.3	±0.986
GalNac-3	*N*-acetylgalactosamine	$0.21x10^7$	0.00098	47.3	±0.984

Table 2- Binding parameters of ssDNA aptamers for their respective targets as derived from surface plasmon resonance spectroscopy data (BIAcore). The targets were the deglycosylated MUC1 tandem repeat MUC1-5TR, the MUC1-5TR-linked Tn antigen and *N*-acetylgalactosamine.

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WE CLAIM:

1. An isolated aptamer that binds to GalNac of the Tn antigen and comprises the sequence at least 80% identical to the sequence consisting of:

AGACTTAGGTGGATGTAGGATCCTT (SEQ ID NO:3);
GAACTTAGGTGGATGTAGGATCCTT (SEQ ID NO:4);
AGACTTAGGTGGATGTAGGATCCAA (SEQ ID NO:5);
GGACTTAGGTGGATGTAGGATCCTT (SEQ ID NO:6);
CTCCGATCCACTAGTAACGGCCGCC (SEQ ID NO:7);
GACCGATCCACTAGTAACGGCCGCC (SEQ ID NO:8);
AGCCGATCCACTAGTAACGGCCGCC (SEQ ID NO:9);
AACCGATCCACTAGTAACGGCCGCC (SEQ ID NO:10);
TACCGATCCACTAGTAACGGCCGCC (SEQ ID NO:11);
GACCGATCCACTAGAAACGGCCGCC (SEQ ID NO:12);
GACCGTACCACTAGTAACGGCCGCC (SEQ ID NO:13);
AAGGGATGACAGGATACGCCAAGCT (SEQ ID NO:14);
AACGCAGTACAGGATACGCCAAGCT (SEQ ID NO:15);
AACCCATGACAGGATACGCCAAGCT (SEQ ID NO:16);
TGCGGATGACAGGATACGCCAAGCT (SEQ ID NO:17); or
TAGGCTAGACAGGATACGCCAAGCT(SEQ ID NO:18).

2. An isolated aptamer that binds to GalNac of the Tn antigen and comprises all or part of the sequence:

AGACTTAGGTGGATGTAGGATCCTT (SEQ ID NO:3);
GAACTTAGGTGGATGTAGGATCCTT (SEQ ID NO:4);
AGACTTAGGTGGATGTAGGATCCAA (SEQ ID NO:5);
GGACTTAGGTGGATGTAGGATCCTT (SEQ ID NO:6);
CTCCGATCCACTAGTAACGGCCGCC (SEQ ID NO:7);
GACCGATCCACTAGTAACGGCCGCC (SEQ ID NO:8);
AGCCGATCCACTAGTAACGGCCGCC (SEQ ID NO:9);
AACCGATCCACTAGTAACGGCCGCC (SEQ ID NO:10);
TACCGATCCACTAGTAACGGCCGCC (SEQ ID NO:11);
GACCGATCCACTAGAAACGGCCGCC (SEQ ID NO:12);

GACCGTACCACTAGTAACGGCCGCC (SEQ ID NO:13);
AAGGGATGACAGGATACGCCAAGCT (SEQ ID NO:14);
AACGCAGTACAGGATACGCCAAGCT (SEQ ID NO:15);
AACCCATGACAGGATACGCCAAGCT (SEQ ID NO:16);
TGCGGATGACAGGATACGCCAAGCT (SEQ ID NO:17); or
TAGGCTAGACAGGATACGCCAAGCT(SEQ ID NO:18).

3. The aptamer according to claim 1 or 2, wherein the aptamer binds to an N-terminal GalNac of the Tn antigen.

4. The aptamer according to any one of claims 1-3 that binds to Tn antigen.

5. The aptamer according to any one of claims 1-4, wherein the aptamer binds to Tn antigen on the surface of a cancer cell or an underglycosylated protein.

6. The aptamer according to claim 5, wherein the underglycosylated protein is a mucin glycoprotein.

7. The aptamer according to claim 6, wherein the mucin glycoprotein is selected from the group consisting of MUC1, MUC2 and MUC5.

8. The aptamer according to claim 5, wherein the cancer is selected from the group consisting of breast, lung, colorectal, gastric, ovarian, colon, prostate, liver and pancreatic carcinomas.

9. The aptamer according to claim 8, wherein the cancer is breast or pancreatic cancer.

10. The aptamer according to any one of claims 1-9 conjugated to a detectable marker, the detectable marker optionally comprising an enzyme, a fluorescent compound, a luminescent material or a radioactive material.

11. The aptamer according to any one of claims 1-9 conjugated to an anticancer agent, the anticancer agent optionally comprising an agent selected from the group consisting of a drug, a radionuclide, a toxin, a cytotoxic peptide, an oligonucleotide and a plasmid comprising a gene encoding a therapeutic agent.

12. The aptamer according to claim 11, wherein the anticancer agent is chlorin e6.

13. Use of the aptamer according to any one of claims 1-10 to detect the presence of a cancer cell or underglycosylated protein in a sample.

14. Use of the aptamer according to claim 11 or 12 to treat or prevent a cancer associated with Tn antigen.

15. Use of the aptamer according to any one of claims 1-9 or 11-12 for preparation of a medicament to treat or prevent a cancer associated with Tn antigen.

16. Use of the aptamer according to any one of claims 1-10 for preparation of a diagnostic agent to detect a cancer associated with Tn antigen.

17. A method of treatment of breast or pancreatic cancer in an animal in need thereof, comprising administering to the animal in need thereof an effective amount of the aptamer of claim 11 or 12.

18. A method of reducing breast or pancreatic cell proliferation in an animal in need thereof, comprising administering to the animal in need thereof an effective amount of the aptamer of claim 11 or 12.

19. A kit comprising the aptamer of any one of claims 1-12 and instructions for use thereof.

20. A method of diagnosing or monitoring a cancer that is associated with Tn antigen comprising:

contacting a sample from a subject with the aptamer according to any one of claims 1-10; and

detecting if a cancer cell or underglycosylated protein is present in the sample by determining whether binding occurs between the aptamer and the sample, wherein binding indicates the presence of a cancer that is associated with Tn antigen.

Figure 1

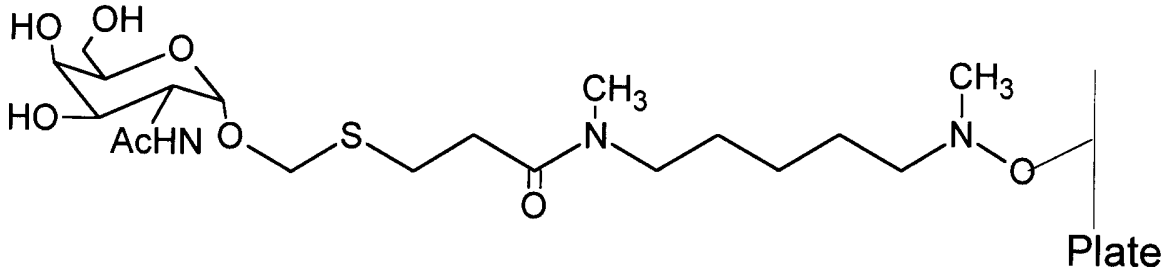


Figure 2

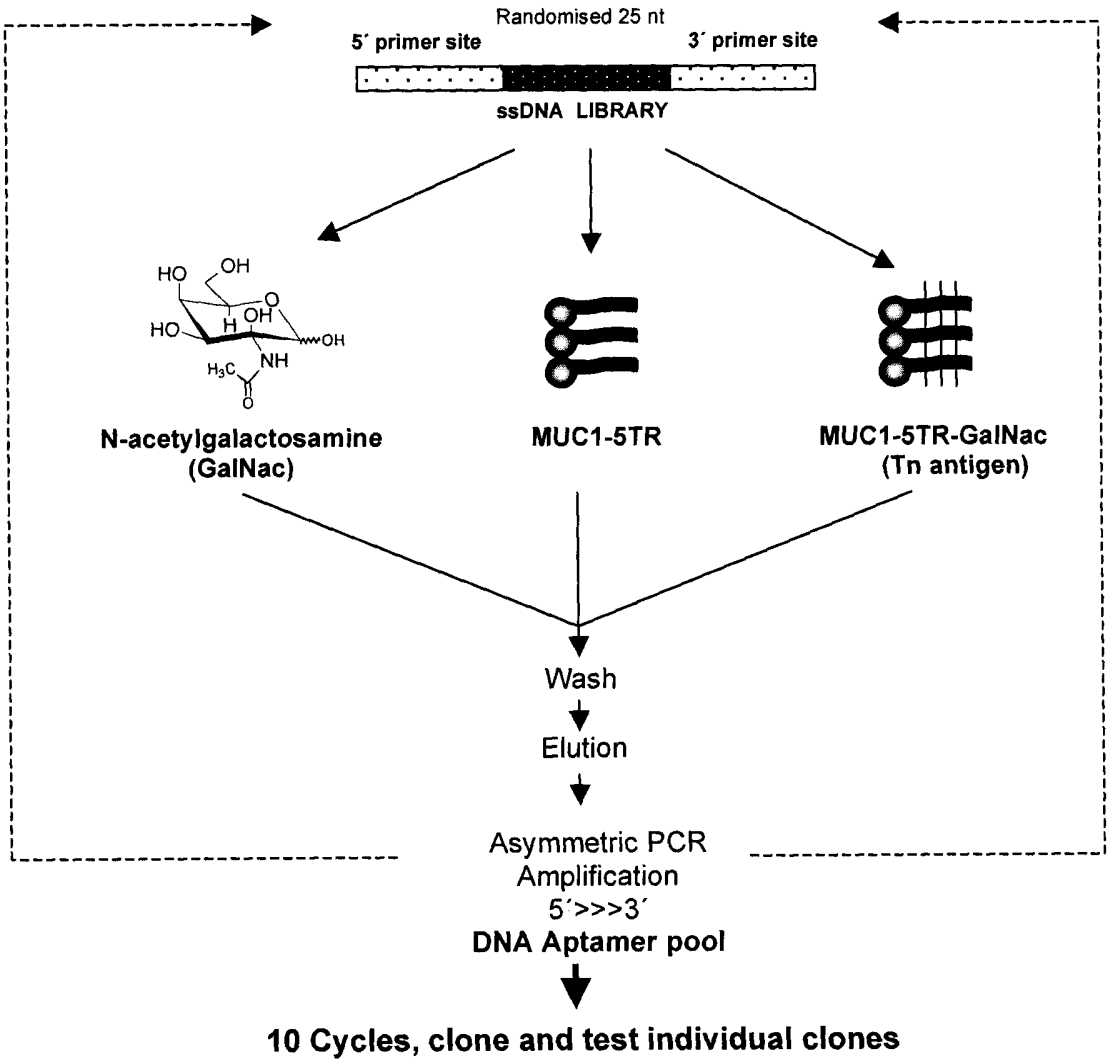

Randomised 25 nt
5′ primer site
3′ primer site
ssDNA LIBRARY
HO
OH
O
H
OH
HO
OH
H3C
NH
O
N-acetylgalactosamine
(GalNac)
MUC1-5TR
MUC1-5TR-GalNac
(Tn antigen)
Wash
Elution
Asymmetric PCR
Amplification
5′>>>3′
DNA Aptamer pool
10 Cycles, clone and test individual clones

Figure 3

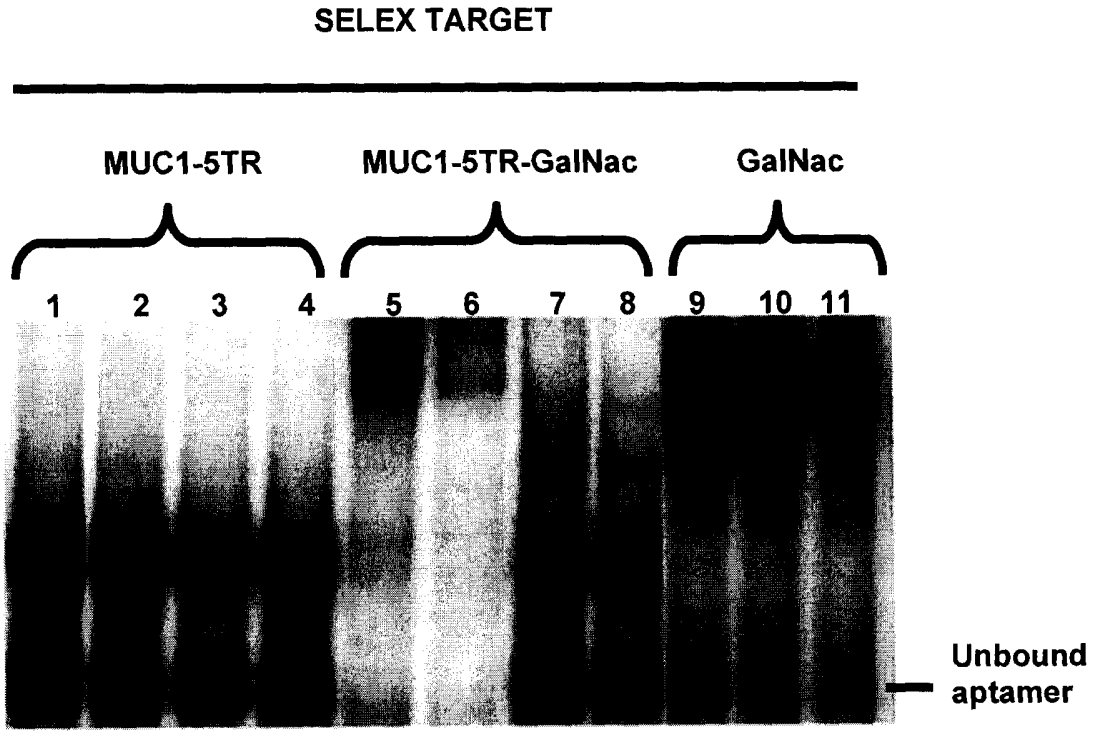

SELEX TARGET
MUC1-5TR
MUC1-5TR-GalNac
GalNac
1
2
3
4
5
6
7
8
9
10
11
Unbound aptamer

Figure 4

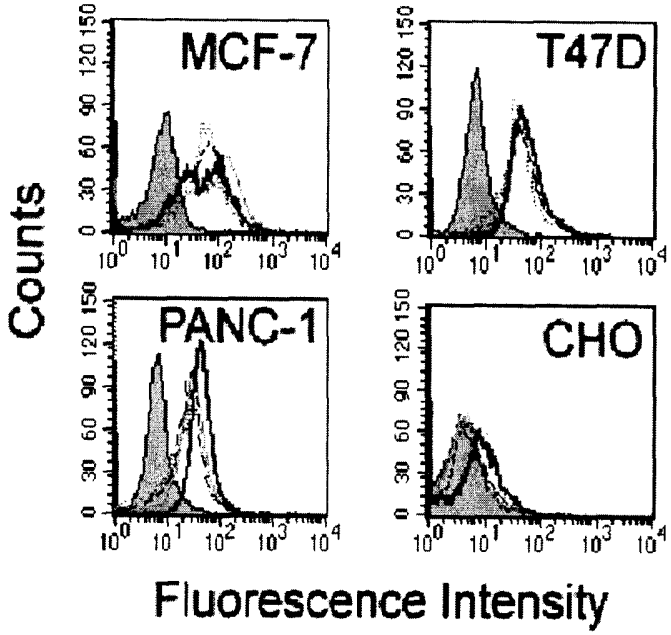


Figure 5

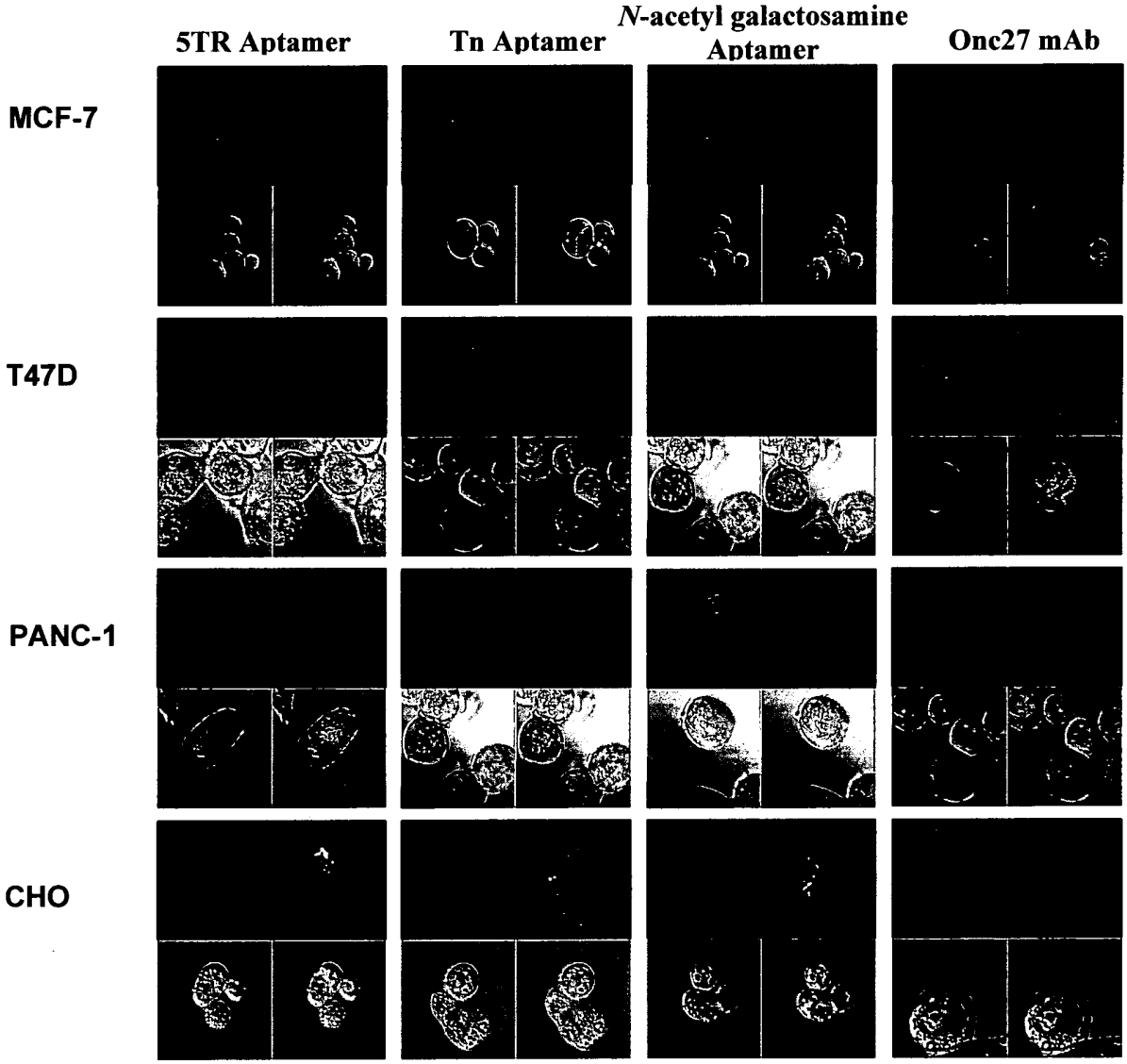

5TR Aptamer
Tn Aptamer
N-acetyl galactosamine Aptamer
Onc27 mAb
MCF-7
T47D
PANC-1
CHO

Figure 6

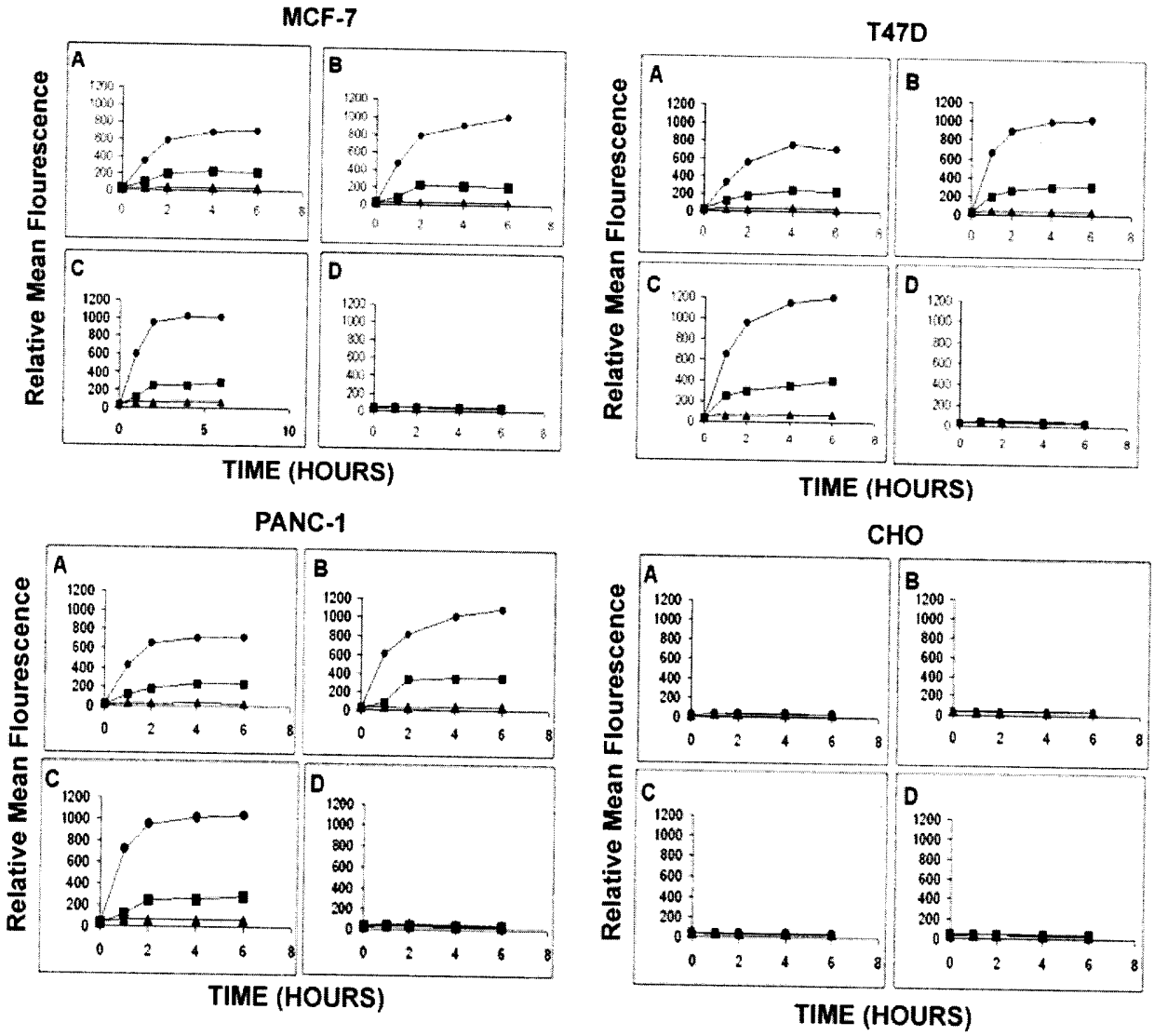

MCF-7
T47D
PANC-1
CHO
A
B
C
D
Relative Mean Flourescence
TIME (HOURS)

Figure 7

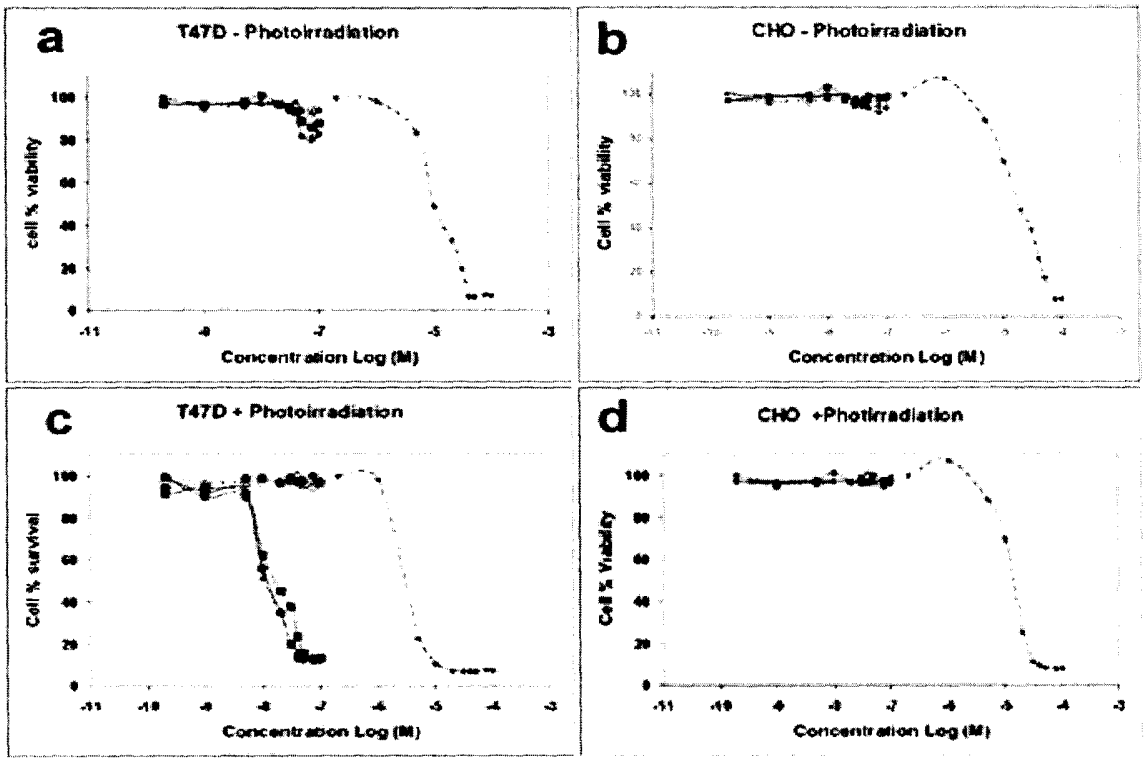

a
T47D - Photoirradiation
cell % viability
Concentration Log (M)
b
CHO - Photoirradiation
Cell % viability
Concentration Log (M)
c
T47D + Photoirradiation
Cell % survival
Concentration Log (M)
d
CHO +Photoirradiation
Cell % Viability
Concentration Log (M)

Figure 8

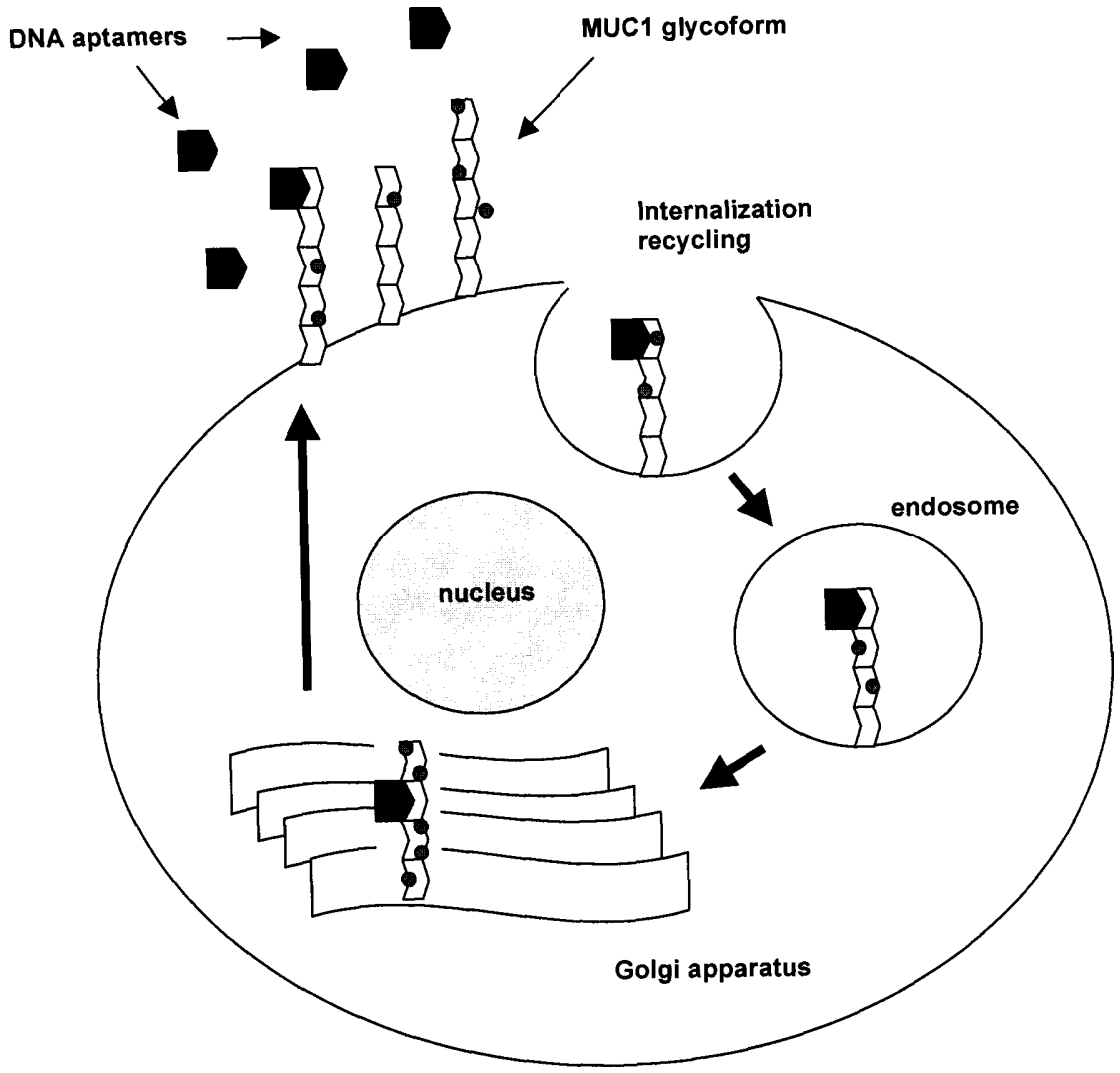
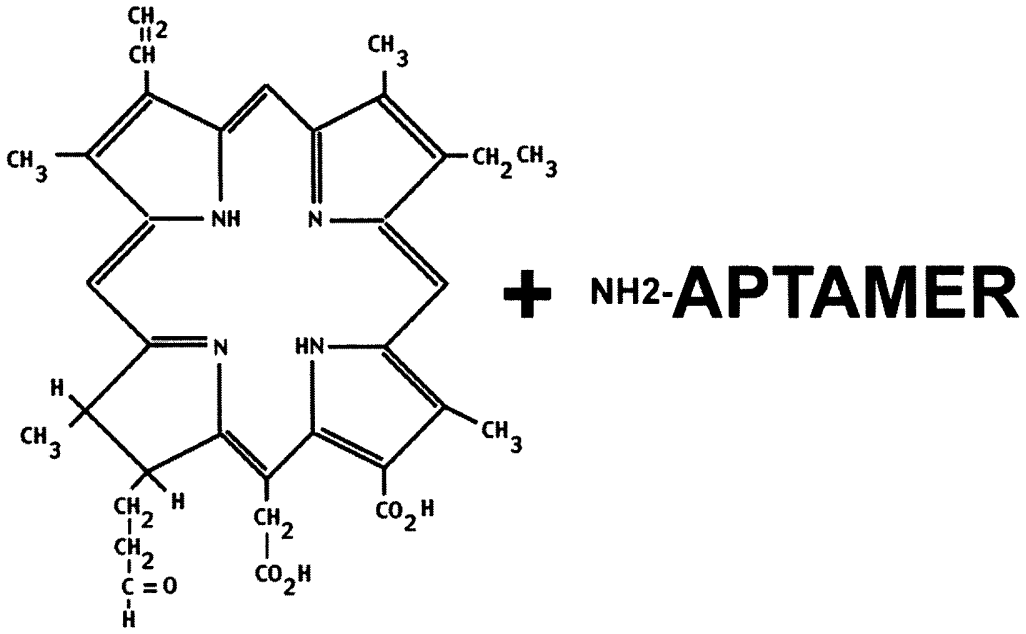

DNA aptamers
MUC1 glycoform
Internalization
recycling
endosome
nucleus
Golgi apparatus

Figure 9


CH2
CH
CH3
CH3
CH2 CH3
NH
N
N
HN
H
CH3
CH3
CH2
H
CH2
CH2
C=O
H
CH2
CO2H
CO2H
+ NH2-APTAMER

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2007/000757

A. CLASSIFICATION OF SUBJECT MATTER
IPC: ***C07H 21/04*** (2006.01) , ***A61K 31/711*** (2006.01) , ***A61P 35/00*** (2006.01) , ***G01N 33/574*** (2006.01)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC: ***C07H 21/04*** (2006.01) , ***A61K 31/711*** (2006.01) , ***A61P 35/00*** (2006.01) , ***G01N 33/574*** (2006.01)

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

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
Delphion, CaPlus, Scopus, Pubmed, Genome Quest, Canadian Patent Database
Keywords: GalNac, cancer, muc, aptamer(s), SELEX, Tn antigen, oligonucleotide(s)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 0192319 A1 (SHANGAI BIOWINDOW GENE DEVELOPMENT INC.), 6 December 2001 (Sequence SEQ ID NO. 4)	1 to 9
X	US 2003/0104410 A1 (AFFYMETRIX, INC.), 5 June 2003 (Sequence SEQ ID NO. 76604)	1 to 9 and 16
X	US 2005/0214823 A1 (AFFYMETRIX, INC.) 29 September 2005 (Sequence SEQ ID NO. 738582)	1 to 9 and 16
X	US 2005/0244851 A1 (AFFYMETRIX, INC.) 3 November 2005 (Sequences SEQ ID Nos: 3362045; 3635463; 3921921; 5990526; 2317357; 3512368; 4033158; 3512368)	1 to 9 and 16

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 July 2007 (10-07-2007)	29 August 2007 (29-08-2007)
Name and mailing address of the ISA/CA Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476	Authorized officer Nathalie Chartrand 819- 994-2341

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2007/000757

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

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

1. [X] Claim Nos. : 17 and 18
because they relate to subject matter not required to be searched by this Authority, namely :

Claims 17 and 18 are directed to a method for treatment of the human or animal body by surgery or therapy which the International Search Authority is not required to search under Rule 39.1(iv) of the PCT. Regardless, this Authority has carried out a search based on the alleged effects or purposes/uses of the product defined in claims 1 to 12.

2. [ ] Claim Nos. :
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically :

3. [ ] Claim Nos. :
because they are dependant claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

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

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

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

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

Remark on Protest

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

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

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2007/000757

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	US 2006/0160121 A1 (WYETH), 20 July 2006 (Sequence 831004)	1 to 9
A	WO 2004/081574 A2 (THE OPEN UNIVERSITY) 23 September 2004 Whole Document	1 to 20
A	HICKE, B.J. et al., "Tumor targeting by an aptamer", THE JOURNAL OF NUCLEAR MEDICINE. April 2006, Vol. 47, No. 4, pages 668-678. Whole Document	1 to 20
A	US 6,699,843 B2 (GILEAD SCIENCES, INC.) 2 March 2004 Whole Document	1 to 20
P, A	FERREIRA, C.S.M. et al., "DNA aptamers that bind to Muc1 tumour marker: design and characterization of Muc1-binding single-stranded DNA aptamers", TUMOR BIOLOGY. October 2006, Vol. 27, pages 289-301. Whole Document	1 to 20

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CA2007/000757

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
WO 0192319A1	06-12-2001	AU 9159601A	11-12-2001
		CN 1324838A	05-12-2001
US 2003104410A1	05-06-2003	None	
US 2005214823A1	29-09-2005	None	
US 2005244851A1	03-11-2005	None	
US 2006160121A1	20-07-2006	AU 2005335216A1	15-02-2007
		CA 2582137A1	15-02-2007
		WO 2007018563A2	15-02-2007
		WO 2007018563A3	21-06-2007
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		EP 1601970A2	07-12-2005
		EP 1806585A1	11-07-2007
		GB 0305422D0	16-04-2003
US 6699843B2*	02-03-2004	AT 343638T	15-11-2006
		AU 7282398A	05-07-1999
		CA 2315271A1	24-06-1999
		DE 69636656D1	07-12-2006
		EP 0828849A1	18-03-1998
		EP 0828849A4	19-01-2005
		EP 1042348A1	11-10-2000
		EP 1042348A4	07-07-2004
		JP 2002508387T	19-03-2002
		US 7141375B2	28-11-2006
		US 2002034506A1	21-03-2002
		US 2004224335A1	11-11-2004
		US 2007021327A1	25-01-2007
		WO 0187351A1	22-11-2001

* The list of members was shortened because of its large number of family members.