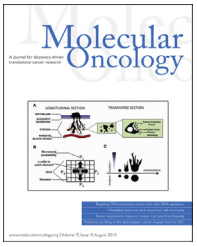
MOLECULAR ONCOLOGY 7 (2013) 799–811

available at www.sciencedirect.com

SciVerse ScienceDirect

www.elsevier.com/locate/molonc



Blocking the attachment of cancer cells *in vivo* with DNA aptamers displaying anti-adhesive properties against the carcinoembryonic antigen

Erik W. Orava[a,c], *Aws Abdul-Wahid*[b,c], *Eric H.-B. Huang*[c], *Amirul Islam Mallick*[b,c], *Jean Gariépy*[a,b,c,*]

[a]*Department of Pharmaceutical Sciences, University of Toronto, Toronto, Ontario M5S 3M2, Canada*
[b]*Department of Medical Biophysics, University of Toronto, Toronto, Ontario M5G 2M9, Canada*
[c]*Physical Sciences, Sunnybrook Research Institute, 2075 Bayview Avenue, Toronto, Ontario M4N3M5, Canada*

ARTICLE INFO

Article history:
Received 18 January 2013
Received in revised form
27 March 2013
Accepted 31 March 2013
Available online 11 April 2013

Keywords:
Adhesion
Homotypic binding
Metastatic cancer
CEA
DNA aptamers

ABSTRACT

The formation of metastatic foci occurs through a series of cellular events, initiated by the attachment and aggregation of cancer cells leading to the establishment of micrometastases. We report the derivation of synthetic DNA aptamers bearing anti-adhesive properties directed at cancer cells expressing the carcinoembryonic antigen (CEA). Two DNA aptamers targeting the homotypic and heterotypic IgV-like binding domain of CEA were shown to block the cell adhesion properties of CEA, while not recognizing other IgV-like domains of CEACAM family members that share strong sequence and structural homologies. More importantly, the pre-treatment of CEA-expressing tumour cells with these aptamers prior to their intraperitoneal implantation resulted in the prevention of peritoneal tumour foci formation. Taken together, these results highlight the effectiveness of targeting the cell adhesion properties of cancer cells with aptamers in preventing tumour implantation.

1. Introduction

Metastatic forms of cancer account for 90% of all cancer-related deaths (Sporn, 1996). Cellular processes associated with tumour cell implantation and expansion of micrometastases at sites distal from a primary tumour site are linked to altered growth signals, deregulation of proliferative potential, evasion of apoptosis/anoikis and cell and matrix adhesion events that create and support the formation of metastatic foci (Fidler, 2003; Hanahan and Weinberg, 2000). As such, surface molecules which mediate intercellular adhesion represent candidate targets for engineering antiadhesive or antiaggregative therapies. One such surface marker is the carcinoembryonic antigen (CEA, CEACAM5 and CD66e), a member of the CEACAM family, an oncofetal antigen overexpressed on the surface of breast, colon, lung and a range of other epithelial cancer cells and an important cancer biomarker (Gold and Freedman, 1965; Goldenberg et al., 1976; Hammarstrom, 1999; Thompson et al., 1991). Under normal physiological conditions, cells lining the colon express CEA in a polarized manner, with low levels of this antigen being detected in the intestinal lumen and blood. In contrast, higher levels of shed CEA are detected in the blood of 95% of patients with colorectal cancer (Chevinsky, 1991; Zhu et al.,

* *Corresponding author*. Department of Pharmaceutical Sciences, University of Toronto, Toronto, Ontario M5S 3M2, Canada. Tel.: +1 416 480 5710.
E-mail address: gariepy@sri.utoronto.ca (J. Gariépy).
1574-7891/$ – see front matter © 2013 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.molonc.2013.03.005

2000). The deregulated overexpression of CEA has been linked to tumour implantation and metastasis (Hammarstrom, 1999).

Membrane-bound CEA is comprised of a 108-amino acid IgV-like N domain followed by 6 Ig-C like domains (A_1, B_1, A_2, B_2, A_3 and B_3) and a 27-amino acid C-terminus region which includes a glycosylphosphatidyl inositol (GPI) anchor signal sequence. The precise biological function of CEA has not been determined, but its deregulated overexpression by cancer cells is associated with a vast array of functional roles such as cooperating with novel oncogenes in cellular transformation, inhibition of anoikis, differentiation inhibition via its GPI anchor, protection of tumour cells to apoptotic stimuli, immunomodulation as well as functioning as an intercellular adhesion molecule displaying both homotypic and heterotypic cell adhesion properties (Benchimol et al., 1989; Jessup et al., 2004; Kitsuki et al., 1995; Ordonez et al., 2000; Screaton et al., 2000, 1997; Soeth et al., 2001).

A common denominator in CEA-dependent adhesion events is its IgV-like N-domain which can lead to cellular aggregation through its binding to itself or CEA IgC-like A_3B_3 domains on distinct tumour cells (defined as homophilic interactions) or its association with extracellular markers (defined as heterophilic interactions) such as its association with $\alpha5\beta1$ integrins in binding to fibronectin (Jessup et al., 1993a; Nicholson and Stanners, 2006; Taheri et al., 2000; Zhou et al., 1993). Clinically, CEA is used to monitor patients with metastatic disease during active therapy, as increasing levels of CEA in serum correlate with treatment failure and poor prognosis (Duffy, 2006; Harris et al., 2007). Importantly, mounting a sustained antibody response directed at an altered self form of the CEA N domain results in the prevention of tumour implantation and formation of metastatic tumour foci in CEA transgenic mice (Abdul-Wahid et al., 2012). This effect has been assigned to the blockage of CEA-dependent adhesion properties by circulating antibodies as well as by immune mechanisms such as antibody-dependent cell cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). Similarly, targeting the CEA IgV-like N domain with cyclised peptides or monoclonal antibodies result in modest blockage of CEA-specific cell adhesion, migration and invasion *in vitro* as well as impeding the metastatic potential in mouse models (Blumenthal et al., 2005; Taheri et al., 2000; Zheng et al., 2011). These findings suggest that CEA-specific, anti-adhesive agents may represent a successful treatment for metastatic cancers linked to the overexpression of CEA.

Aptamers represent an emerging alternative to protein-based ligands. Specifically, aptamers are short single-stranded DNA or RNA oligonucleotides that adopt complex secondary and tertiary structures that allow for their specific and high affinity binding to a range of targets that include metal ions, proteins, bacterial cells and tumour cells (Hamula et al., 2008; Hicke et al., 2001; Morris et al., 1998; Rajendran and Ellington, 2008). Aptamers are derived through an iterative selection process, termed systematic evolution of ligands by exponential enrichment (SELEX), using a synthetic library containing a randomized region of 25–80 nucleotides flanked by two constant regions for PCR amplification (Tuerk and Gold, 1990). RNA aptamers are more labile than DNA oligonucleotides and the cost as well as time required to perform RNA SELEX searches are greater. More stable variants of RNA aptamers can be assembled with a modified T7 polymerase to incorporate 2′-fluoro and 2′-O-Me nucleotides.

Our group has reported the expression and purification of a folded recombinant form of the IgV-like N domain able to elicit an immune response as well as recapitulate the binding property of glycosylated full length CEA with CEA-expressing cells and purified human CEA from cancer patients. Importantly, the un-glycosylated form of the CEA N domain represents a suitable target for identifying aptamers since this domain has few putative glycosylation sites and that glycosylation of the N domain does not contribute to the adhesive properties between CEA N domain molecules (Charbonneau and Stanners, 1999; Krop-Watorek et al., 2002). We report the isolation of two functional DNA aptamers selected to bind this recombinant form of the IgV-like N domain of CEA and show its ability to block CEA-mediated cellular interactions and inhibit peritoneal tumour nodule formation from CEA-expressing tumour cells *in vivo*.

2. Materials and methods

2.1. Generation of recombinant CEA modules

Recombinant CEA (rCEA) modules N, FLAG-N and A_3B_3 were expressed and purified as previously reported (Abdul-Wahid et al., 2012). Briefly, recombinant CEA domains were purified from inclusion bodies in *Escherichia coli* under denaturing conditions using urea (8 M). The protein was subsequently purified by nickel-NTA chromatography and treated with Detoxigel (endotoxin removing gel; Thermo Fisher Scientific Inc.) to remove remaining traces of bacterial lipopolysaccharides (LPS).

2.2. Aptamer selection and cloning

The initial ssDNA library contained a central randomized sequence of 25 nucleotides flanked by two primer regions with the sequence 5′ GAC GAT AGC GGT GAC GGC ACA GAC G-(25N)-CGT ATG CCG CTT CCG TCC GTC GCT C 3′. The forward primer 5′ GAC GAT AGC GGT GAC GGC ACA GAC G 3′ and reverse primer 5′ GAG CGA CGG ACG GAA GCG GCA TAC G 3′ were used for selection and cloning (IDT Technologies, Inc.). A 50 nmol aliquot of the library was first counter-selected against Ni-NTA magnetic beads prior to selection against rCEA N in order to reduce non-specifically bound DNA species. The resulting sub-library was then exposed to 10 μg of His-tagged rCEA N domain immobilized onto Ni-NTA beads suspended in 1 ml of Selection Buffer (150 mM NaCl, 50 mM Tris pH 8.0) at 37 °C for 1 h. Unbound DNA oligonucleotides were washed away with a 10-fold excess of selection buffer and DNA-protein complexes were eluted from the recovered beads using an imidazole containing buffer (Selection buffer with 240 mM imidazole). The ssDNA component was precipitated with sodium perchlorate/isopropanol and recaptured using a silica membrane-based purification system (Qiagen Inc., Mississauga, Ontario).

The DNA aptamers were then amplified by asymmetrical PCR using a 10-fold excess of forward primer. After every three subsequent rounds of selection, the amount of target was reduced in half to increase the selection pressure to capture the tightest binding species. After 12 rounds of selection, the bound sequences were amplified by PCR to produce double stranded products, cloned into a pCR4-TOPO TA vector (Invitrogen) and sequences were analysed using BioEdit sequence alignment editor software (Ibis Therapeutics, Carlsbad, USA).

2.3. Aptamer-based inhibition of CEA homotypic interactions

An enzyme-linked immunosorbent assay (ELISA)-based binding assay was employed to identify aptamers capable of inhibiting homotypic interactions between FLAG-tagged rCEA N domain and either rCEA A_3B_3 or rCEA N. Briefly, 96-well flat-bottomed Falcon microtiter plates (Becton–Dickinson Biosciences, Franklin Lakes, NJ) were coated with either N or A_3B_3 domain (1 μg/well in 100 μl) in coating buffer (0.2 M carbonate/bicarbonate, pH 9.4) at 37 °C. Plates were then blocked with BSA (1% in PBS) and salmon sperm DNA (200 μl; 10 μg/ml) for 1 h at room temperature then aptamers were added (200 μl; 25 μg/ml, 1 μM) overnight at 4 °C in PBS-T (0.05% Tween-20). Plates were then washed three times with PBS-T and FLAG-tagged rCEA N domain was added (100 μl; 10 μg/ml, 670 nM) for 1 h at room temperature after being incubated with a given aptamer (100 μl; 25 μg/ml, 1 μM) for 1 h at room temperature. After wash steps with PBS-T, the remaining bound FLAG-tagged rCEA N was detected by incubating the plates for 1 h at room temperature with horseradish peroxidase (HRP) coupled anti-FLAG monoclonal antibody M2 (1:2500 dilution; Sigma-Aldrich). All experiments were performed in quadruplicate.

2.4. Cells lines and growth conditions

The murine colonic carcinoma cell lines MC38.CEA and MC38 were kindly provided by Dr. Jeffrey Schlom (National Cancer Institute, Bethesda, Maryland). The cervical adenocarcinoma cell line HeLa (ATCC No CCL-2) as well as transfected cell lines $HeLa^{CEACAM1}$, $HeLa^{CEACAM3}$, $HeLa^{CEACAM5}$, $HeLa^{CEACAM6}$ and $HeLa^{CEACAM8}$ used for flow cytometry experiments were a gift from Dr. Scott Gray-Owen (University of Toronto, Toronto, Canada). All cell lines were cultured at 37 °C, 5.0% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and dihydrostreptomycin (100 μg/ml).

2.5. Aptamer-based inhibition of homophilic cellular adhesion

The ability of aptamers to inhibit CEA-dependent cellular adhesion was measured in real-time using an xCELLigence RTCA SP label-free, impedance-based cell sensing device (Roche Applied Sciences, Laval, Canada). The inhibition of CEA-dependent cellular adhesion was monitored using MC38.CEA and MC38 cells (2.5×10^4 cells per well) grown in complete medium as described above. Cell suspensions were dispensed alone, with aptamers (100 μl, 250 μg/ml, 10 μM) or in the presence of the rCEA N domain acting as a positive control (100 μl, 50 μg/ml, 3.3 μM) into wells of a 96-well microtiter plate incorporating a sensor electrode array (E-plates) that had been precoated with the rCEA N domain, rCEA A_3B_3 domain or BSA (1 μg/well). Cell attachment was quantified as a change in relative impedance, termed cell index (CI) (Matrone et al., 2010). The adhesion of MC38.CEA cells in the absence of aptamers served as a positive control. Data was collected after 3 h to allow cells to fully adhere to protein-coated plates but before the start of cell proliferation. All experiments were performed in duplicate and were repeated three times.

2.6. Aptamer binding to CEA on cells as measured by flow cytometry

The binding specificity of aptamers N54 and N56 to the N domain of CEA was assessed by flow cytometry using the cell lines MC38.CEA (CEA^+) and MC38 (CEA^-) as well as HeLa cells expressing different members of the CEACAM family. Aptamers N54, N56 and the control aptamer cApt were synthesized with a 5′end Cy5 fluorophore (IDT Technologies, Inc., Coralville, Iowa). Cells were grown to mid-log phase and detached using an enzyme-free EDTA based cell dissociation buffer (Sigma-Aldrich, St. Louis, MO) washed with PBS ($-CaCl_2$, $-MgCl_2$) and resuspended at a concentration of 10^6 cells/ml in cold PBS. Aptamers were then added to 1.0×10^6 cells at a final concentration of 200 nM in 1 ml. The expression of CEACAMs on HeLa transfected cells lines was confirmed using a FITC-labelled polyclonal anti-CEACAM antibody (Gift from Dr. Gray-Owen, University of Toronto, Toronto, Canada). CEA expression was confirmed with a CEA-specific COL-1 antibody (Invitrogen Inc.). Aptamers and antibody were allowed to bind for 2 h at 4 °C. Cells were then washed three times in cold PBS and subsequently analysed by flow cytometry using a FACScan (BD Biosciences, Franklin Lakes, NJ).

2.7. Inhibition of MC38.CEA tumour implantation

For tumour implantation studies, 5.0×10^5 MC38.CEA cells were co-injected in the intraperitoneal cavity of C57BL/6 mice with either aptamers N54, N56, N71, cApt or no aptamer (saline) (200 μl; 2.5 mg/ml, 100 μM). After 21 days, mice were sacrificed and the number of nodules and their volumes were recorded following dissection, as previously described (Abdul-Wahid et al., 2012). Specifically, the length and width of tumour nodules were measured using microcallipers. Tumour volumes were calculated using the modified formula where the volume of the tumour (mm^3) equals $[(x^2 \times y)/2]$; where x and y represent the transverse and longitudinal diameters of the tumour respectively. Each group consisted of 3 females and 2 male mice. All animals were kept under standard pathogen-free conditions at the Ontario Cancer Institute animal facility. Experiments were performed under the approval of the local animal welfare committee and in accordance with the rules and regulations of the Canadian Council for Animal Care.

2.8. Aptamer cytotoxicity assay

MC38.CEA cells were seeded for 24 h before cell viability experiments were performed in 96-well flat-bottom microtiter plates at a density of 5.0×10^3 cells/well in DMEM medium containing 10% FBS. Aptamers at a concentration of either 250 μg/ml (10 μM) or 2.5 mg/ml (100 μM) were incubated with the cells in medium for 24 h at a volume of 100 μl. Cells were then washed with warm PBS and incubated in complete medium for another 24 h. The viability of adherent cells was subsequently determined using a sulforhodamine B assay (Skehan et al., 1990). The absorbance of the sulforhodamine B signal in each well was read at 570 nm using a plate reader. Each experiment was performed in quadruplicate and repeated three times.

2.9. Analysis of aptamer-based innate immune responses

The aptamers N54 (200 μl; 500 μg/ml, 20 μM) and cApt (200 μl; 500 μg/ml, 20 μM) as well as a TLR9 ligand CpG ODN (5′-TCCATGACGTTCCTGACGTT-3′); type B murine, ODN 1826, Invivogen, CA; (200 μl; 50 μg/ml, 500 nM) were dissolved in sterile USP saline. These oligonucleotides (200 μl) were administered intraperitoneally to 6–8 weeks old C57BL/6 mice. An untreated group of mice received an injection of 200 μl of saline alone. Three hours after injection, mice were sacrificed and their serum was collected for analysis. Serum IL-8 and TNFα concentrations were determined using the DuoSet ELISA development kits for mouse CXCL1/KC (murine IL-8, R & D systems Inc.) and murine TNFα (R & D systems Inc.) as outlined by the manufacturer.

2.10. Statistical methods and data analysis

Data sets derived from individual groups of mice were compared using Student-t-test and grouped data sets were analysed by ANOVA. Statistical analyses and graphs were assembled using GraphPad PRISM (version 5.01, GraphPad software for Science, San Diego, CA). P values ≤ 0.05 were considered significant unless otherwise indicated. Flow cytometry statistics were analysed using WinMDI (version 2.8, Windows multiple document interface for flow cytometry, Scripps research institute).

3. Results

3.1. Generation of DNA aptamers displaying inhibitory properties towards CEA-dependent homotypic adhesion events

Short, 25-base long DNA aptamer sequences specifically recognizing a recombinant protein coding for residues 1–132 of mature CEA (referred to as rCEA N domain) were identified by the SELEX approach (Figure 1). Specifically, twelve iterative rounds of PCR-based selection were performed yielding six unique DNA aptamer sequences (Figure 1A). These six

A

Aptamer	GACGATAGCGGTGACGGCACAGACG-Sequence-GTATGCCGCTTCCGTCCGTCGCTC
N54	TCCCGCATCCTCCGCCGTGCCGACCC
N56	CCCCAGGAAGAACCTACTCACTGATC
N57	CACCGCTCTTATGCCACCATTTTCAC
N59	CCGCTACCCCATCCACGCCAATCCC
N65	CCCGCATCCTCCGCCGTGCTGACCCC
N71	AAATCCCCGCTGAATTACCACTTTAC
cApt	AGTCAGTCAGTCAGTCAGTCAGTCA

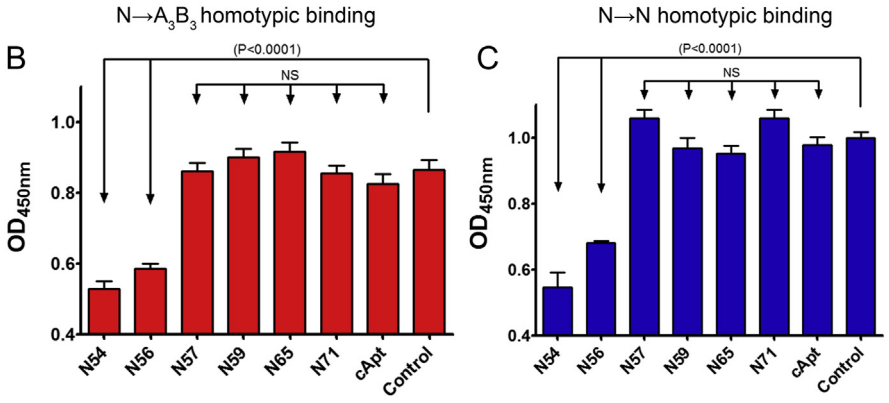


Figure 1 – Aptamers selected to bind to the CEA IgV-like N domain inhibit homotypic adhesion events. (A) Aptamer sequences identified using the SELEX procedure. CEA N-domain-specific aptamers inhibit homotypic binding events. ELISA plates (96-well) were pre-coated with (B) rCEA A_3B_3 or with (C) the rCEA N domain. Aptamers selected to the N-domain and control aptamer (cApt) were added to the wells, incubated overnight at 4 °C, washed followed by the addition of FLAG-tagged rCEA N incubated with aptamers for 1 h and added to the appropriate wells. Bound FLAG-tagged rCEA N was detected using an anti-FLAG HRP-coupled M2 mAb. Each bar represents the average percent of binding ± SEM ($n = 4$).

sequences, labelled N54, N56, N57, N59, N65 and N71, as well as a control aptamer (cApt) were synthesized with their primer regions and subsequently tested using an ELISA-based assay to assess their ability to directly block the binding of the IgV-like N domain to either rCEA IgC-like A_3B_3 domains (Figure 1B) or to itself (to rCEA N domain; Figure 1C). Aptamers N54 and N56 were the only aptamers found to display inhibitory properties of both types of binding events, where N54 inhibited 39% of the rCEA N → rCEA A_3B_3 signal and 45% of the rCEA N → rCEA N signal relative to control wells (in the absence of aptamer). Aptamer N56 inhibited 32% of the signal associated with either homotypic interactions (Figure 1B and C). Interestingly, aptamer N65 shares a nearly identical sequence to N54 with the exception of two bases flanking the ends of the sequence and a C to T substitution in the sequence [5′ GCTGAC 3′]. This finding suggests that the antiadhesive property of N54 is sensitive to even a single base change in its sequence.

3.2. *Aptamers N54 and N56 inhibit homophilic cellular adhesion*

CEA-dependent homophilic interactions are a prerequisite to the expansion of metastatic tumour foci. As such, the inhibitory capacity of aptamers N54 and N56 was determined using the CEA-expressing murine cell line MC38.CEA and its parental CEA-negative cell line MC38. MC38.CEA cells adhere to the wells of impedence-based plates (E-plates) pre-coated with the rCEA N and A_3B_3 domains, but only weakly to wells coated with BSA (Figure 2A). Addition of aptamers N54 and N56 resulted in a loss of 59% and 49% of the signal arising from homophilic cellular adhesion between the immobilized CEA N domain and MC38.CEA cells respectively after 3 h. This time point was chosen as it was observed that cells were able to fully adhere without the presence of inhibitors (Supplementary Figure 1). Similarly, a 45% and 51% decrease in signal was observed for MC38.CEA cells interacting with the immobilized rCEA A_3B_3. In contrast, the control aptamers cApt and N71 showed no significant inhibition of homophilic interactions (Figure 2A). As a positive control, the soluble rCEA N domain was pre-incubated with MC38.CEA cells and resulted in a 59% and 44% loss of binding signal of these cells to the immobilized rCEA N or A_3B_3 domain respectively. As expected, aptamer treatments as well as BSA and rCEA N domain showed no ability to inhibit MC38 cells from adhering to rCEA N- or BSA-coated plates (Figure 2B). Subsequently, the inhibitory effects of aptamers N54 and N56 (as 75-base long

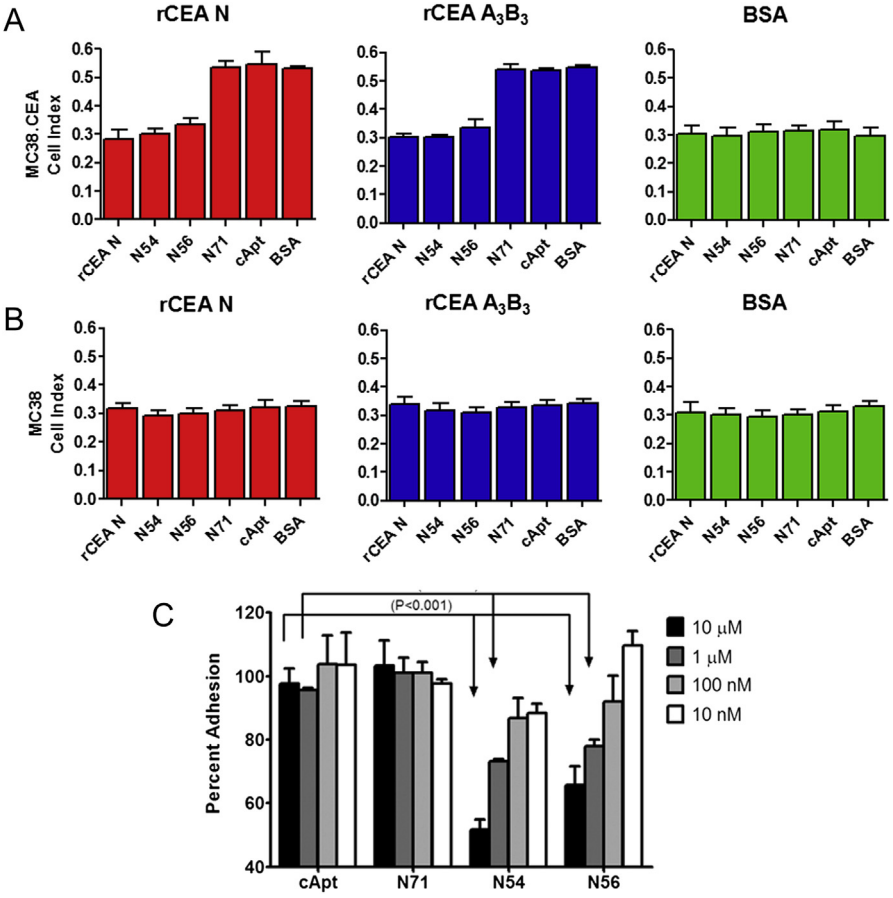


Figure 2 – Addition of aptamers specific to the CEA N domain inhibits homophilic cellular adhesion. (A) Addition of aptamers N54 and N56 significantly inhibited CEA-dependent binding of MC38.CEA cells to wells coated with either rCEA N domain or rCEA A_3B_3 while not affecting their binding to BSA coated wells. (B) Addition of aptamers N54 and N56 had no effect on CEA$^-$ MC38 cells adhering to wells coated with rCEA N, rCEA A_3B_3 or BSA. (C) Dose-dependent inhibition of CEA-mediated cellular adhesion of MC38.CEA to rCEA N coated wells was monitored in the presence of increasing concentrations of specific and control aptamers. Each bar in Panels A and B represents the average cell index values observed ± SEM ($n = 6$). In panel C, each bar represents the average percentage of cell binding ± SEM ($n = 6$).

aptamers) on cell adhesion were titrated in a dose-dependent manner (Figure 2C). A significant inhibition of MC38.CEA adhesion was observed starting at an aptamer concentration of 1 μM. Control aptamers cApt and N71 showed no effect suggesting that the inhibition of homophilic cellular adhesion by aptamers N54 and N56 was not due to a concentration dependent non-specific effect.

To further characterize the inhibitory properties of aptamers N54 and N56, we constructed series of truncated forms to determine the minimal binding regions required to retain their inhibition of CEA-dependent homophilic adhesion (Figure 3A). Aptamer N54 did not retain its ability to inhibit cellular adhesion after a total of 18 bases were removed from both ends of its sequence (aptamer N54-57; Figure 3B). Interestingly, although full length N56 was not as effective as N54 in inhibiting MC38.CEA cell adherence (36% compared to 48% inhibition respectively), N56 did retain its inhibitory ability when truncated down to 32 bases with no significant decrease in the adherence of cells as compared to the full length sequence (Figure 3B). Further truncations of N56 however yielded inactive inhibitors of cell adhesion (results not shown).

3.3. *Aptamers N54 and N56 specifically recognize the N domain of CEA*

The IgV-like N domain of CEA is homologous in sequence to that of other CEACAM members. Specifically, the alignment of CEACAM1, CEACAM3, CEACAM5 (CEA), CEACAM6, and CEACAM8 IgV-like N domain primary structures indicate that 61% of residues along their sequences are identical with up to 84% of residues being similar (Figure 4A). In addition, the known structures of the CEACAM1, CEACAM5 and CEACAM8 N domains also indicate that these IgV-like N domains adopt the same folded structure (Figure 4B) (Fedarovich et al., 2006; Korotkova et al., 2008). Accordingly, the ability of aptamers N54 and N56 to specifically recognize the N domain of CEA and not IgV-like N domains of related CEACAMs was assessed by monitoring the binding of Cy5-labelled aptamers to CEACAM^{+} and CEACAM^{-} cells by flow cytometry. Specifically, HeLa cells were stably transfected to express CEACAM1, CEACAM3, CEACAM5, CEACAM6 or CEACAM8.

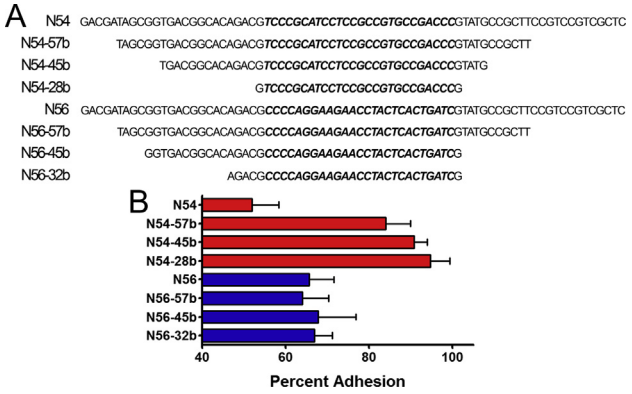


Figure 3 – Minimal regions required for aptamer inhibition of homophilic cellular adhesion. (A) List of aptamer sequences synthesized to determine the minimum aptamer regions needed to inhibit CEA-dependent binding of cells to immobilized CEA N domain. Bold italic letters represent the variable region of aptamer sequences identified by SELEX searches. (B) Inhibition of CEA-dependent binding of MC38.CEA cells to rCEA N with full length and truncated aptamer N54 and N56 sequences. Each bar represents the average percentage of cell binding to wells ± SEM ($n = 6$).

Analysis of the CEACAM-expressing HeLa cells as well as the CEA^{+} MC38.CEA cells, CEA^{-} HeLa and MC38 cells demonstrated that aptamers N54 and N56 specifically bound to MC38.CEA and HeLa^{CEACAM5} cells while the irrelevant cApt control aptamer showed no binding to any of the cells tested (Figure 5). The FITC-labelled polyclonal anti-CEACAM antibody confirmed the expression of individual CEACAMs in all transfected HeLa cell lines and the CEA antibody COL-1 confirmed the presence of CEA (Figure 5). Aptamer N56 binding to MC38.CEA and HeLa^{CEACAM5} resulted in a ~7-fold increase in mean fluorescence signal intensity (Figure 5B and D). Aptamer N54 binding to MC38.CEA and HeLa^{CEACAM5} resulted in a greater increase in binding as shown by a ~16-fold and ~14-fold increase in mean fluorescence intensities respectively (Figure 5B and D). Similar binding patterns were seen for the endogenously CEA expressing cell lines MCF-7, HT29 and BxPC3 (Supplementary Figure 2). The binding of Cy5-labelled aptamers N54 and N56 to MC38.CEA cells was also determined as a function of concentration (Figure 5, panel I). Using a single site binding model, it was calculated that aptamers N54 and N56 display binding constants (K_d) of 45 ± 11 nM and 78 ± 24 nM respectively to their CEA target on MC38.CEA cells. Together, these findings suggest that the derived N54 and N56 aptamer sequences specifically bind their cognate target with high affinity.

3.4. *Addition of aptamers N54 and N56 to MC38.CEA cells reduces tumour implantation* in vivo

The ability of aptamers N54 and N56 to inhibit CEA-dependent tumour implantation and subsequent metastasis was addressed by monitoring their ability to interfere with the implantation of murine MC38.CEA tumour cells in the peritoneal cavity of C57BL/6 mice. Briefly, murine MC38.CEA cells were pretreated for 30 min at 37 °C with 500 μg (200 μl; 100 μM) of control aptamers cApt, N71, inhibitory aptamers N54, N56 or left untreated and co-injected directly into the peritoneal cavity of mice (Figure 6A). Mice were then euthanized after 21 days to assess tumour implantation by recording the number of tumour nodules as well as their volumes. Post-mortem analyses of the dissected mice showed that tumour masses were limited to the peritoneal cavity, and that tumour nodules were numerous in the control animal groups given either no aptamer, cApt or N71 (Figure 6B and C). In contrast, implanted MC38.CEA cells treated with aptamers N54 and N56 generated significantly fewer tumour nodules (Figure 6B). Specifically, aptamer N56 reduced tumour implantation as seen by an average decrease of 48% of cumulative tumour volume while N54 had a significant decrease of 57% compared to untreated mice (Figure 6C). Control aptamers cApt and N71 showed no significant decrease in tumour implantation in relation with the control group that was just implanted with MC38.CEA cells (untreated). The inhibitory effect of aptamers N54 and N56 was even more dramatic in terms of implantation when tumour nodules were enumerated and compared to control

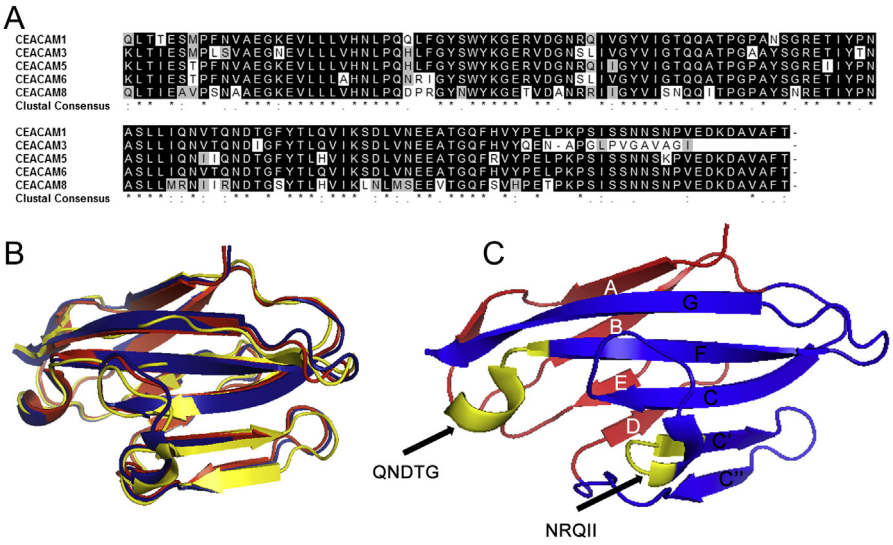


Figure 4 – Alignment of the IgV-like N-domains of CEACAM family members. (A) Sequence alignment of the N-domain of CEACAM family members involved in cell–cell interactions. (B) Structural alignment of the N-domains of CEACAM1 (red; PDB: 2GK2), CEA (blue; PDB: 2QSQ) and CEACAM8 (yellow; PDB:2DKS). (C) The ABED face (red) and the CFG face (blue) of CEA. Residues 42 to 46 (NRQII) and residues 80 to 84 (QNDTG) are shown in yellow. Structures were obtained from the protein data bank (PDB) with accession numbers shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

groups (Figure 6D). Specifically, in the group pretreated with aptamer N54, four of five mice did not develop a secondary tumour nodule compared to untreated groups which had an average of ~6 tumour nodules. Mice treated with control aptamer cApt and N71 showed no significant decrease in the number of tumour foci when compared to the aptamer-untreated group.

3.5. *Aptamers specific to the CEA N domain are not cytotoxic and aptamer N54 does not activate an innate immune response*

Cytotoxicity studies on MC38.CEA cells treated with aptamers and rCEA N were conducted using the sulforhodamine B cell viability assay to determine if the decrease in tumour implantation seen was due to a cytotoxic effect of the aptamers (Matthews et al., 1987). MC38.CEA cells were incubated in DMEM complete medium for 24 h in the presence of either 250 μg/ml of aptamer (100 μl; 10 μM, same as used for *in vitro* experiments) or 2.5 mg/ml (100 μl; 100 μM) which represented the concentration of aptamer pretreated MC38.CEA cells prior to tumour implantation (Figure 7A). None of the aptamers incubated with MC38.CEA cells led to a loss of cell viability at these concentrations which suggests that the reduction in tumour implantation observed for N54 and N56 was not due to aptamer-based cytotoxicity towards MC38.CEA cells.

In view of its potency as an inhibitor of CEA-mediated cell adhesion, we also tested whether aptamer N54 could induce an innate immune response similar to oligodeoxynucleotides containing CpG motifs which promote T_h1 responses by signalling through TLR9. Mice received an i.p. injection of 100 μg (200 μl; 20 μM) of control aptamer (cApt), aptamer N54 or 10 μg (100 μl; 500 nM) of a known TLR9 ligand CpG ODN and the amount of TNFα and mouse IL-8 present in their serum was quantified after 3 h. Activation of TLR9 with the CpG ODN increased the serum levels of mouse IL-8 by ~45-fold while aptamers cApt N54 yielded statistically non-significant increases in serum IL-8 (Figure 7B). Similar results were observed in terms of increased production of serum TNFα by CpG ODN but not by aptamers cApt and N54 (Figure 7C). These results suggest that N54 does not activate an *in vivo* innate immune response associated with the secretion of inflammatory cytokines.

4. Discussion

Cancer cells typically display molecular signatures on their surface which have been exploited more recently as targets for aptamers to chaperone therapeutic cargos into cells (Orava et al., 2010). In the present study, functional DNA aptamers were developed against the key homotypic binding domain of CEA generated from *E. coli*. We selected DNA aptamers, as opposed to RNA aptamers, because they are more stable [less susceptible to hydrolysis], less expensive and more easily derived by SELEX approaches than RNA aptamers. Functional aptamers acting as mimics of molecules linked to cellular signalling pathways have been reported and in some instances shown to block events as diverse as angiogenesis, thrombosis, viral replication and inflammatory responses (Bless et al., 1997; Bock et al., 1992; Boiziau et al., 1999; Chou et al., 2005; Muller et al., 2009; Ng et al., 2006; Paborsky et al., 1993; Schneider et al., 1995). In the context of adhesion, several aptamers have previously been reported which bind to cell surface glycoproteins known as selectins (Huang et al., 1997; Schmidmaier and Baumann, 2008).

Specifically, a phosphorothioate-modified aptamer to E-selectin named ESTA-1 bound with nanomolar affinity and

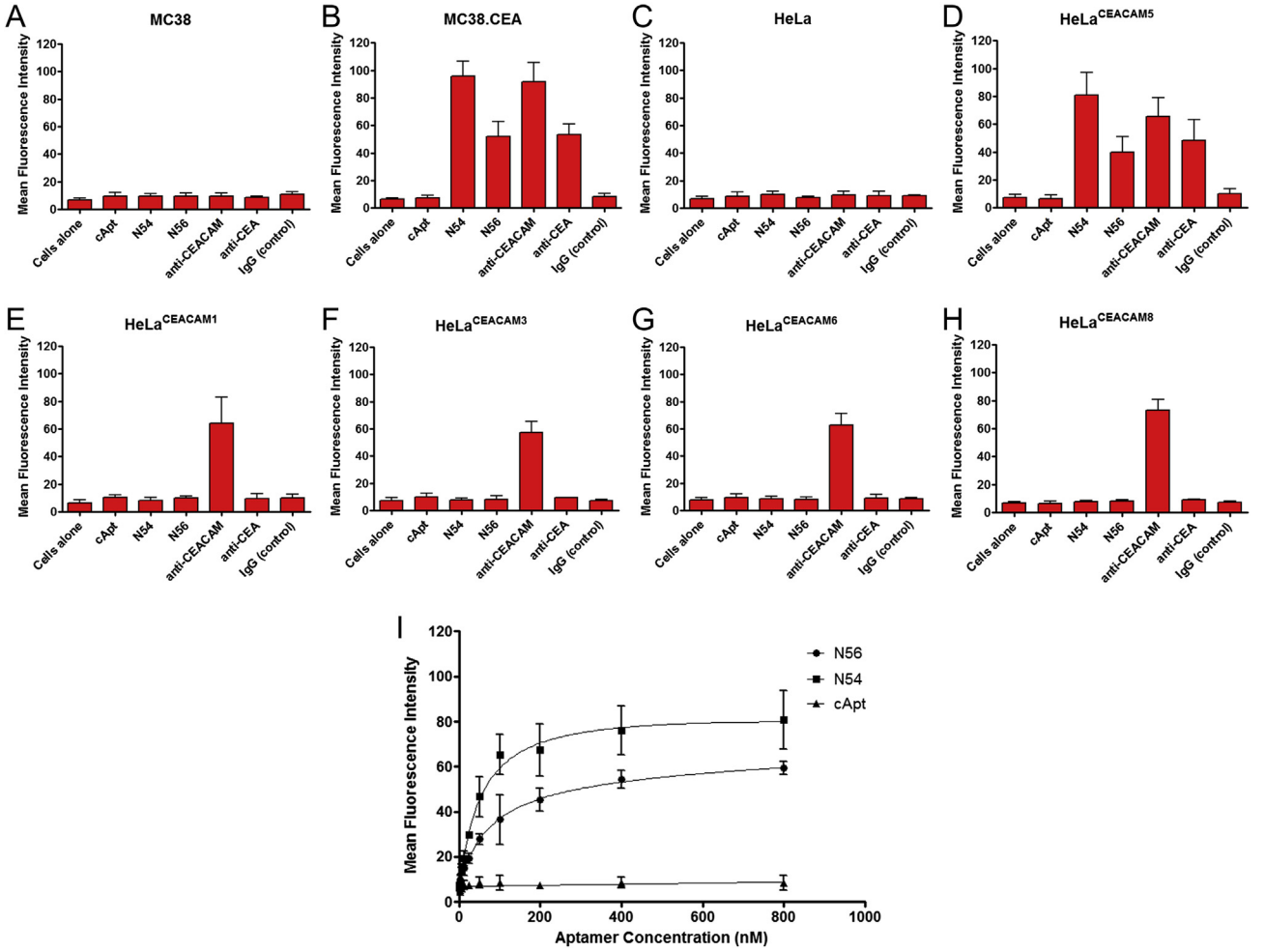


Figure 5 – Aptamer binding to CEA$^+$ and CEA$^-$ cells using Cy5 labelled aptamers by flow cytometry. Cy5-labelled aptamers N54, N56 and cApt were incubated with CEA$^-$ cells (A) MC38, (C) HeLa or CEA$^+$ (B) MC38.CEA and (D) HeLa^{CEACAM5}. Aptamers N54 and N56 did not detect the presence of other CEACAM family members as shown by low mean fluorescence intensities observed for (E) HeLa^{CEACAM1}, (F) HeLa^{CEACAM3}, (G) HeLa^{CEACAM6}, (H) HeLa^{CEACAM8} transfected cell lines. A FITC-labelled polyclonal anti-CEACAM antibody was used to confirm the surface expression of CEACAM proteins and an anti-CEA mAb to monitor for the presence of CEA. The auto-fluorescence signal arising from unlabelled cells is shown for cells alone. (I) Binding of Cy5-labelled aptamers N54, N56 and cApt to MC38.CEA cells. Data points represent average mean fluorescence intensity values ± SEM ($n = 3$).

inhibited over 75% of sialyl lewis X positive cells from adhering to endothelial cells overexpressing E-selectin *in vitro* (Mann et al., 2010). As well, an RNA aptamer to P-selectin known as ARC5690 which contained 2′-fluoro pyrimidine and 2′-methoxy purines inhibited the adhesion of sickle red blood cells and leukocytes to endothelial cells by 90% and 80% respectively in a sickle cell disease model *in vivo* (Gutsaeva et al., 2010). Furthermore, a DNA aptamer to L-selectin inhibited L-selectin-mediated rolling of lymphocyte and neutrophils on activated endothelial cells *in vitro* as well as blocking lymphocyte trafficking to the lymph nodes *in vivo* (Hicke et al., 1996). The present study highlights functional DNA aptamers able to specifically inhibit CEA-expressing tumour cells from forming metastastic tumour foci. Importantly, CEA plays a key role in tumour progression and the establishment of metastatic foci by CEA-expressing tumours (Berinstein, 2002). CEA has been shown to function as an intercellular adhesion molecule, as a function of reciprocal homophilic binding between N and A_3 domains (Jessup et al., 1993a; Taheri et al., 2000; Zhou et al., 1993), an attribute that contributes to its tumorigenicity (Camacho-Leal and Stanners, 2008; Samara et al., 2007). Specifically, its involvement in homotypic and heterotypic interactions correlate with the level of implantation and proliferation of CEA-expressing tumours at distal sites such as the lungs, liver and peritoneal cavity (Asao et al., 1991; Samara et al., 2007; Zhou et al., 1993; Zimmer and Thomas, 2001). CEA molecules bind in a homotypic manner by virtue of the interaction between their N and A_3 domains of opposing CEA molecules, events which results in the formation of a network of homophilic cellular contacts between CEA-expressing cells causing cell aggregation, implantation and invasion of organs (Hostetter et al., 1990; Jessup et al., 1993b; Zhou et al., 1993; Zimmer and Thomas, 2001). CEA has also been shown to

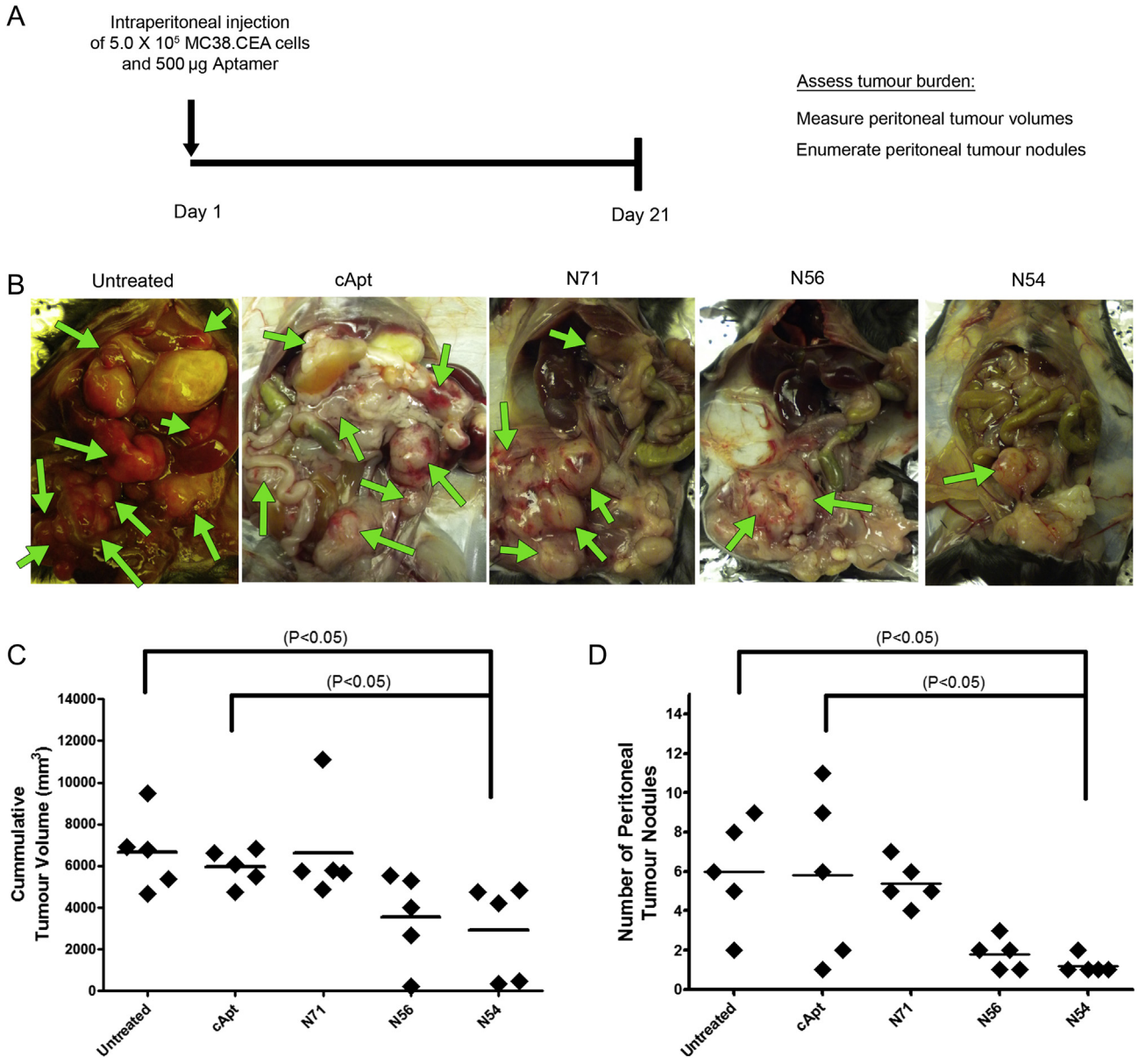


Figure 6 – Pre-treatment of CEA-expressing MC38.CEA cancer cells with CEA-specific aptamers reduces tumour implantation *in vivo*. (A) Experimental design of implantation studies. MC38.CEA cells (5×10^5) were co-administered intraperitoneally into C57BL/6 mice with aptamers. Untreated cells served as a positive control for tumour implantation. Animals were sacrificed 21 days post implantation. (B) Photographs illustrating the presence of MC38.CEA tumour nodules in the peritoneal cavity at Day 21 post-implantation. (C) Cumulative tumour volumes in each group of mice ($n = 5$) after 21 days. (D) The number of tumour nodules in the peritoneal cavity in each treatment group ($n = 5$) after 21 days post-implantation.

heterotypically interact with additional binding partners (Benchimol et al., 1989; Oikawa et al., 1989). We hypothesized that blocking the homotypic binding of CEA would represent an effective strategy for inhibiting its adhesive behaviour.

Using the CEA IgV-like N domain as a target for aptamer selection using the SELEX process, we identified two unique aptamers, termed N54 and N56 that possess the ability to inhibit CEA-mediated homotypic interactions (Figure 1). Of the two aptamers, N54 displayed a moderately greater ability to inhibit these interactions that was comparable to tumour-neutralizing antisera derived from mice vaccinated with the rCEA N domain (Abdul-Wahid et al., 2012). Addition of N54 significantly inhibited the binding of murine CEA-expressing MC38.CEA cells to wells coated with rCEA N domain yet had no effect on CEA$^-$ MC38 cells adhering to plates coated with rCEA N (Figure 2A and B). These results demonstrate that aptamers N54 and N56 are able to effectively block the homotypic interaction between the CEA N domains and CEA N domain to rCEA A_3B_3.

Aptamers N54 and N56 differ greatly in the minimal regions needed to inhibit homophilic cellular adhesion. Aptamer N54 requires both its primer and variable regions as the deletion

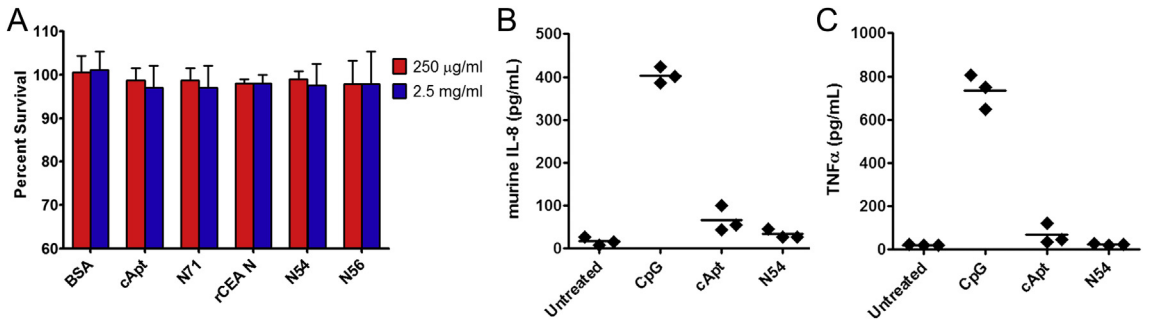


Figure 7 – Aptamer N54 is noncytotoxic and does not activate innate immune responses (A) A sulforhodamine B cell viability assay was performed on MC38.CEA cells treated with DNA aptamers or rCEA N at the concentration used for *in vivo* studies (red bars) or at a 10-fold higher concentration (blue bars). Aptamer N54, cApt or CpG (positive control) were injected intraperitoneally into C57/BL6 mice. The animals were sacrificed after 3 h for analysis of TLR-9 dependent activation of (B) IL-8 and (C) TNF-α secretion ($n = 3$).

of a total of 18 bases from its 3′and 5′ ends resulted in a loss of its inhibition of CEA-mediated cell adhesion from ~50% to ~16% relative to the full length sequence (Figure 3A and B). However, aptamer N56 can be truncated from 75 bases down to 32 bases while retaining its inhibitory function (Figure 3A and B). Further deletions to N56 however resulted in a loss of its inhibition of CEA cell adhesion function (data not shown).

Several members of the CEACAM family have been shown to be involved in cell–cell interactions sharing a high level of sequence identity within their N domain with CEA. Yet, aptamers N54 and N56 were able to uniquely bind to the N domain of CEA (Figures 4 and 5). Structurally, the N domain of CEACAM1, CEAMCAM3, CEA, CEACAM6 and CEACAM8 adopt an identical IgV-like fold displaying defined faces. The N domain of CEACAMs displays two faces: an ABED face and an opposite CFG face (Korotkova et al., 2008; Taheri et al., 2000). The CFG interface of CEACAMs has been shown to mediate CEACAM–CEACAM interactions (Figure 4C) (Markel et al., 2004). Furthermore, peptides corresponding to residues 42 to 46 (NRQII) and residues 80 to 84 (QNDTG) on CEA were found to modestly block CEA-mediated cellular aggregation (Taheri et al., 2000) at concentrations >150-fold higher than aptamers N54 and N56. Both of these peptide sequences are found on the CFG face of CEA (Taheri et al., 2000). Interestingly, the peptide NRQII corresponding to residues 42 to 46 is unique to CEA while the sequence QNDTG is found in CEACAM1 and CEACAM6. In view of the specificity of aptamers N54 and N56 in binding to CEA, it would suggest that these aptamers may interact with residues in the vicinity of peptide NRQII on the CFG face of the CEA N domain.

One issue facing the use DNA aptamers as therapeutics is their short half-life *in vivo* due to nuclease degradation and their rapid clearance through the kidneys (based on their low molecular weights). The circulation half-lives of aptamers can be increased by conjugating them to polyethylene glycol (PEG) moieties. As well, their stability in serum can be increased by substituting nucleotides with modifications of either sugar residues (eg. 2′ OH group with 2′-amino, 2′-fluoro 2′-O-methyl), the phosphate (eg. Phosphorothioate) or of the base (2′-thiopyrimidine, methyl or trifluoromethyl) (Healy et al., 2004; Latham et al., 1994; Mayer, 2009; Schoetzau et al., 2003; Willis et al., 1998). Also, Locked Nucleic acids (LNA) have been introduced within their structures to increase stability (Barciszewski et al., 2009). However, the *in vivo* tumour implantation mouse model used in this study to assess the ability of aptamers N54 and N56 to inhibit the adhesion and proliferation of murine MC38.CEA cells in the peritoneal cavity of mice were performed with no modification to the aptamer structure. Both of these aptamers were effective in inhibiting tumour implantation in the peritoneal model (Figure 6B). Interestingly, an RNA aptamer to CEA has recently been reported that prevents hepatic metastasis (Lee et al., 2012). However, this RNA aptamer differs from the present DNA apamers N54 and N56 in two key aspects. First, this RNA aptamer prevents the binding of CEA to death receptor 5 (DR5), thus contributing to the prevention of hepatic metastases by inducing anoikis. In contrast, aptamers N54 and N56 are non-cytotoxic towards MC38.CEA cells and the treatment of MC38.CEA cells with aptamers N54 and N56 did not lead to growth arrest/reduction (SRB cell viability assay) in relation to untreated cells (Figure 7A). Secondly, the reported RNA aptamer to CEA was shown to bind the PELPK motif present at residues 108–122 of CEA. This motif is present on CEACAM1 and CEACAM 6: two broadly-expressed CEACAMs on normal tissues, suggesting that this aptamer may not be specific for CEA (possible off-target effects). In contrast, aptamers N54 and N56 showed specific binding to CEA suggesting that their anti-adhesive effects focus on the specific inhibition of homotypic CEA interactions (Figure 4). Importantly, the murine cell line MC38.CEA used for both *in vitro* and *in vivo* studies, does not express other human CEACAM members on its surface. In view of the specificity of aptamer N54 and N56 for the N domain of CEA only, these aptamers are not expected to inhibit possible homophilic cellular interactions involving other CEACAM members present on tumour cells.

Finally, DNA aptamers are generally considered to be non-immunogenic (Foy et al., 2007; Yu et al., 2009). However, to confirm that aptamer N54, as an example, did not generate an inflammatory innate immune response as seen for CpG ODN (a ligand for TLR-9), mice were given via an intraperitoneal injection, a bolus of either an irrelevant aptamer (cApt), aptamer N54 or a positive control CpG ODN, and their sera analysed after 3 h for the production of inflammatory cytokines IL-8 and TNFα (Figure 7B and C). As projected, aptamer

N54 did not generate a serum increase in CpG ODN-associated cytokines suggesting that this aptamer is non-immunogenic.

In summary, this study reports the identification of two DNA aptamers that are able to specifically recognize the N domain of the cancer-associated antigen CEA and block its homophilic adhesive properties. These aptamers specifically bound to the IgV-like N domain of CEA, with a dissociation constant in the nanomolar range and significantly inhibited tumour implantation of murine MC38.CEA cells by virtue of their antiadhesive properties. As well, aptamer N54 displayed no cytotoxicity towards MC38.CEA cells and did not trigger a TLR-9 dependent innate immune response.

5. Conclusion

Tumour foci formation leading to metastases represents the major cause of death in cancer patients. There is a need to develop new concepts and therapies that can halt or control the establishment or expansion of secondary tumour foci. CEA represents a logical target for designing new therapies as it is over-expressed on many epithelial cancer tissues and serves key roles in cellular aggregation processes and attachment to extracellular matrix elements. This report provides the first, direct evidence that aptamer-based, CEA-directed, anti-adhesive strategies can block metastatic foci formation *in vivo*.

Acknowledgements

This work was financially supported by the Canadian Breast Cancer Foundation. E.O. is the recipient of a Canadian Institutes of Health Research Training Program in Biological Therapeutics Studentship Award. The authors wish to thank Dr. J. Schlom for providing the MC38.CEA and MC38 cell lines, Dr. S. Gray-Owen for providing the CEACAM-transfected HeLa cell lines as well as Drs. Kaoru Geddes and Dana Philpott for their help with immune monitoring assays.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molonc.2013.03.005.

REFERENCES

Abdul-Wahid, A., Huang, E.H., Lu, H., Flanagan, J., Mallick, A.I., Gariepy, J., 2012. A focused immune response targeting the homotypic binding domain of the carcinoembryonic antigen blocks the establishment of tumor foci in vivo. Int. J. Cancer 131, 2839–2851.

Asao, T., Fukuda, T., Yazawa, S., Nagamachi, Y., 1991. Carcinoembryonic antigen levels in peritoneal washings can predict peritoneal recurrence after curative resection of gastric cancer. Cancer 68, 44–47.

Barciszewski, J., Medgaard, M., Koch, T., Kurreck, J., Erdmann, V.A., 2009. Locked nucleic acid aptamers. Methods Mol. Biol. 535, 165–186.

Benchimol, S., Fuks, A., Jothy, S., Beauchemin, N., Shirota, K., Stanners, C.P., 1989. Carcinoembryonic antigen, a human tumor marker, functions as an intercellular adhesion molecule. Cell 57, 327–334.

Berinstein, N.L., 2002. Carcinoembryonic antigen as a target for therapeutic anticancer vaccines: a review. J. Clin. Oncol. 20, 2197–2207.

Bless, N.M., Smith, D., Charlton, J., Czermak, B.J., Schmal, H., Friedl, H.P., Ward, P.A., 1997. Protective effects of an aptamer inhibitor of neutrophil elastase in lung inflammatory injury. Curr. Biol. 7, 877–880.

Blumenthal, R.D., Hansen, H.J., Goldenberg, D.M., 2005. Inhibition of adhesion, invasion, and metastasis by antibodies targeting CEACAM6 (NCA-90) and CEACAM5 (Carcinoembryonic Antigen). Cancer Res. 65, 8809–8817.

Bock, L.C., Griffin, L.C., Latham, J.A., Vermaas, E.H., Toole, J.J., 1992. Selection of single-stranded DNA molecules that bind and inhibit human thrombin. Nature 355, 564–566.

Boiziau, C., Dausse, E., Yurchenko, L., Toulme, J.J., 1999. DNA aptamers selected against the HIV-1 trans-activation-responsive RNA element form RNA-DNA kissing complexes. J. Biol. Chem. 274, 12730–12737.

Camacho-Leal, P., Stanners, C.P., 2008. The human carcinoembryonic antigen (CEA) GPI anchor mediates anoikis inhibition by inactivation of the intrinsic death pathway. Oncogene 27, 1545–1553.

Charbonneau, J., Stanners, C.P., 1999. Role of carbohydrate structures in CEA-mediated intercellular adhesion. Cell. Adhes. Commun. 7, 233–244.

Chevinsky, A.H., 1991. CEA in tumors of other than colorectal origin. Semin. Surg. Oncol. 7, 162–166.

Chou, S.H., Chin, K.H., Wang, A.H., 2005. DNA aptamers as potential anti-HIV agents. Trends Biochem. Sci. 30, 231–234.

Duffy, M.J., 2006. Serum tumor markers in breast cancer: are they of clinical value? Clin. Chem. 52, 345–351.

Fedarovich, A., Tomberg, J., Nicholas, R.A., Davies, C., 2006. Structure of the N-terminal domain of human CEACAM1: binding target of the opacity proteins during invasion of Neisseria meningitidis and N. gonorrhoeae. Acta Crystallogr. D Biol. Crystallogr. 62, 971–979.

Fidler, I.J., 2003. The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. Nat. Rev. Cancer 3, 453–458.

Foy, J.W., Rittenhouse, K., Modi, M., Patel, M., 2007. Local tolerance and systemic safety of pegaptanib sodium in the dog and rabbit. J. Ocul. Pharmacol. Ther. 23, 452–466.

Gold, P., Freedman, S.O., 1965. Specific carcinoembryonic antigens of the human digestive system. J. Exp. Med. 122, 467–481.

Goldenberg, D.M., Sharkey, R.M., Primus, F.J., 1976. Carcinoembryonic antigen in histopathology: immunoperoxidase staining of conventional tissue sections. J. Natl. Cancer Inst. 57, 11–22.

Gutsaeva, D.R., Parkerson, J.B., Yerigenahally, S.D., Kurz, J.C., Schaub, R.G., Ikuta, T., Head, C.A., 2010. Inhibition of cell adhesion by anti-P-selectin aptamer: a new potential therapeutic agent for sickle cell disease. Blood 117, 727–735.

Hammarstrom, S., 1999. The carcinoembryonic antigen (CEA) family: structures, suggested functions and expression in normal and malignant tissues. Semin. Cancer Biol. 9, 67–81.

Hamula, C.L., Zhang, H., Guan, L.L., Li, X.F., Le, X.C., 2008. Selection of aptamers against live bacterial cells. Anal. Chem. 80, 7812–7819.

Hanahan, D., Weinberg, R.A., 2000. The hallmarks of cancer. Cell 100, 57–70.

Harris, L., Fritsche, H., Mennel, R., Norton, L., Ravdin, P., Taube, S., Somerfield, M.R., Hayes, D.F., Bast Jr., R.C., 2007. American Society of Clinical Oncology 2007 update of recommendations for the use of tumor markers in breast cancer. J. Clin. Oncol. 25, 5287–5312.

Healy, J.M., Lewis, S.D., Kurz, M., Boomer, R.M., Thompson, K.M., Wilson, C., McCauley, T.G., 2004. Pharmacokinetics and biodistribution of novel aptamer compositions. Pharm. Res. 21, 2234–2246.

Hicke, B.J., Marion, C., Chang, Y.F., Gould, T., Lynott, C.K., Parma, D., Schmidt, P.G., Warren, S., 2001. Tenascin-C aptamers are generated using tumor cells and purified protein. J. Biol. Chem. 276, 48644–48654.

Hicke, B.J., Watson, S.R., Koenig, A., Lynott, C.K., Bargatze, R.F., Chang, Y.F., Ringquist, S., Moon-McDermott, L., Jennings, S., Fitzwater, T., Han, H.L., Varki, N., Albinana, I., Willis, M.C., Varki, A., Parma, D., 1996. DNA aptamers block L-selectin function in vivo. Inhibition of human lymphocyte trafficking in SCID mice. J. Clin. Invest. 98, 2688–2692.

Hostetter, R.B., Augustus, L.B., Mankarious, R., Chi, K.F., Fan, D., Toth, C., Thomas, P., Jessup, J.M., 1990. Carcinoembryonic antigen as a selective enhancer of colorectal cancer metastasis. J. Natl. Cancer Inst. 82, 380–385.

Huang, Y.W., Baluna, R., Vitetta, E.S., 1997. Adhesion molecules as targets for cancer therapy. Histol. Histopathol 12, 467–477.

Jessup, J.M., Kim, J.C., Thomas, P., Ishii, S., Ford, R., Shively, J.E., Durbin, H., Stanners, C.P., Fuks, A., Zhou, H., et al., 1993a. Adhesion to carcinoembryonic antigen by human colorectal carcinoma cells involves at least two epitopes. Int. J. Cancer 55, 262–268.

Jessup, J.M., Laguinge, L., Lin, S., Samara, R., Aufman, K., Battle, P., Frantz, M., Edmiston, K.H., Thomas, P., 2004. Carcinoembryonic antigen induction of IL-10 and IL-6 inhibits hepatic ischemic/reperfusion injury to colorectal carcinoma cells. Int. J. Cancer 111, 332–337.

Jessup, J.M., Petrick, A.T., Toth, C.A., Ford, R., Meterissian, S., O'Hara, C.J., Steele Jr., G., Thomas, P., 1993b. Carcinoembryonic antigen: enhancement of liver colonisation through retention of human colorectal carcinoma cells. Br. J. Cancer 67, 464–470.

Kitsuki, H., Katano, M., Morisaki, T., Torisu, M., 1995. CEA-mediated homotypic aggregation of human colorectal carcinoma cells in a malignant effusion. Cancer Lett. 88, 7–13.

Korotkova, N., Yang, Y., Le Trong, I., Cota, E., Demeler, B., Marchant, J., Thomas, W.E., Stenkamp, R.E., Moseley, S.L., Matthews, S., 2008. Binding of Dr adhesins of Escherichia coli to carcinoembryonic antigen triggers receptor dissociation. Mol. Microbiol. 67, 420–434.

Krop-Watorek, A., Klopocki, A.G., Czerwinski, M., Lisowska, E., 2002. Adhesive properties of carcinoembryonic antigen glycoforms expressed in glycosylation-deficient Chinese hamster ovary cell lines. Acta Biochim. Pol 49, 273–283.

Latham, J.A., Johnson, R., Toole, J.J., 1994. The application of a modified nucleotide in aptamer selection: novel thrombin aptamers containing 5-(1-pentynyl)-2'-deoxyuridine. Nucleic Acids Res. 22, 2817–2822.

Lee, Y.J., Han, S.R., Kim, N.Y., Lee, S.H., Jeong, J.S., Lee, S.W., 2012. An RNA aptamer that binds carcinoembryonic antigen inhibits hepatic metastasis of colon cancer cells in mice. Gastroenterology 143, 155–165 e158.

Mann, A.P., Somasunderam, A., Nieves-Alicea, R., Li, X., Hu, A., Sood, A.K., Ferrari, M., Gorenstein, D.G., Tanaka, T., 2010. Identification of thioaptamer ligand against E-selectin: potential application for inflamed vasculature targeting. PLoS One 5.

Markel, G., Gruda, R., Achdout, H., Katz, G., Nechama, M., Blumberg, R.S., Kammerer, R., Zimmermann, W., Mandelboim, O., 2004. The critical role of residues 43R and 44Q of carcinoembryonic antigen cell adhesion molecules-1 in the protection from killing by human NK cells. J. Immunol. 173, 3732–3739.

Matrone, M.A., Whipple, R.A., Thompson, K., Cho, E.H., Vitolo, M.I., Balzer, E.M., Yoon, J.R., Ioffe, O.B., Tuttle, K.C., Tan, M., Martin, S.S., 2010. Metastatic breast tumors express increased tau, which promotes microtentacle formation and the reattachment of detached breast tumor cells. Oncogene 29, 3217–3227.

Matthews, N., Neale, M.L., Jackson, S.K., Stark, J.M., 1987. Tumour cell killing by tumour necrosis factor: inhibition by anaerobic conditions, free-radical scavengers and inhibitors of arachidonate metabolism. Immunology 62, 153–155.

Mayer, G., 2009. The chemical biology of aptamers. Angew. Chem. Int. Ed. Engl. 48, 2672–2689.

Morris, K.N., Jensen, K.B., Julin, C.M., Weil, M., Gold, L., 1998. High affinity ligands from in vitro selection: complex targets. Proc. Natl. Acad. Sci. U. S. A. 95, 2902–2907.

Muller, J., Isermann, B., Ducker, C., Salehi, M., Meyer, M., Friedrich, M., Madhusudhan, T., Oldenburg, J., Mayer, G., Potzsch, B., 2009. An exosite-specific ssDNA aptamer inhibits the anticoagulant functions of activated protein C and enhances inhibition by protein C inhibitor. Chem. Biol. 16, 442–451.

Ng, E.W., Shima, D.T., Calias, P., Cunningham Jr., E.T., Guyer, D.R., Adamis, A.P., 2006. Pegaptanib, a targeted anti-VEGF aptamer for ocular vascular disease. Nat. Rev. Drug Discov. 5, 123–132.

Nicholson, T.B., Stanners, C.P., 2006. Specific inhibition of GPI-anchored protein function by homing and self-association of specific GPI anchors. J. Cell. Biol. 175, 647–659.

Oikawa, S., Inuzuka, C., Kuroki, M., Matsuoka, Y., Kosaki, G., Nakazato, H., 1989. Cell adhesion activity of non-specific cross-reacting antigen (NCA) and carcinoembryonic antigen (CEA) expressed on CHO cell surface: homophilic and heterophilic adhesion. Biochem. Biophys. Res. Commun. 164, 39–45.

Orava, E.W., Cicmil, N., Gariepy, J., 2010. Delivering cargoes into cancer cells using DNA aptamers targeting internalized surface portals. Biochim. Biophys. Acta 1798, 2190–2200.

Ordonez, C., Screaton, R.A., Ilantzis, C., Stanners, C.P., 2000. Human carcinoembryonic antigen functions as a general inhibitor of anoikis. Cancer Res. 60, 3419–3424.

Paborsky, L.R., McCurdy, S.N., Griffin, L.C., Toole, J.J., Leung, L.L., 1993. The single-stranded DNA aptamer-binding site of human thrombin. J. Biol. Chem. 268, 20808–20811.

Rajendran, M., Ellington, A.D., 2008. Selection of fluorescent aptamer beacons that light up in the presence of zinc. Anal. Bioanal. Chem. 390, 1067–1075.

Samara, R.N., Laguinge, L.M., Jessup, J.M., 2007. Carcinoembryonic antigen inhibits anoikis in colorectal carcinoma cells by interfering with TRAIL-R2 (DR5) signaling. Cancer Res. 67, 4774–4782.

Schmidmaier, R., Baumann, P., 2008. ANTI-ADHESION evolves to a promising therapeutic concept in oncology. Curr. Med. Chem. 15, 978–990.

Schneider, D.J., Feigon, J., Hostomsky, Z., Gold, L., 1995. High-affinity ssDNA inhibitors of the reverse transcriptase of type 1 human immunodeficiency virus. Biochemistry 34, 9599–9610.

Schoetzau, T., Langner, J., Moyroud, E., Roehl, I., Vonhoff, S., Klussmann, S., 2003. Aminomodified nucleobases: functionalized nucleoside triphosphates applicable for SELEX. Bioconjug. Chem. 14, 919–926.

Screaton, R.A., DeMarte, L., Draber, P., Stanners, C.P., 2000. The specificity for the differentiation blocking activity of carcinoembryonic antigen resides in its glycophosphatidyl-inositol anchor. J. Cell. Biol. 150, 613–626.

Screaton, R.A., Penn, L.Z., Stanners, C.P., 1997. Carcinoembryonic antigen, a human tumor marker, cooperates with Myc and Bcl-2 in cellular transformation. J. Cell. Biol. 137, 939–952.

Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J.T., Bokesch, H., Kenney, S., Boyd, M.R., 1990. New colorimetric cytotoxicity assay for anticancer-drug screening. J. Natl. Cancer Inst. 82, 1107–1112.

Soeth, E., Wirth, T., List, H.J., Kumbhani, S., Petersen, A., Neumaier, M., Czubayko, F., Juhl, H., 2001. Controlled ribozyme targeting demonstrates an antiapoptotic effect of carcinoembryonic antigen in HT29 colon cancer cells. Clin. Cancer Res. 7, 2022–2030.

Sporn, M.B., 1996. The war on cancer. Lancet 347, 1377–1381.

Taheri, M., Saragovi, U., Fuks, A., Makkerh, J., Mort, J., Stanners, C.P., 2000. Self recognition in the Ig superfamily. Identification of precise subdomains in carcinoembryonic antigen required for intercellular adhesion. J. Biol. Chem. 275, 26935–26943.

Thompson, J.A., Grunert, F., Zimmermann, W., 1991. Carcinoembryonic antigen gene family: molecular biology and clinical perspectives. J. Clin. Lab. Anal. 5, 344–366.

Tuerk, C., Gold, L., 1990. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. Science 249, 505–510.

Willis, M.C., Collins, B.D., Zhang, T., Green, L.S., Sebesta, D.P., Bell, C., Kellogg, E., Gill, S.C., Magallanez, A., Knauer, S., Bendele, R.A., Gill, P.S., Janjic, N., 1998. Liposome-anchored vascular endothelial growth factor aptamers. Bioconjug. Chem. 9, 573–582.

Yu, D., Wang, D., Zhu, F.G., Bhagat, L., Dai, M., Kandimalla, E.R., Agrawal, S., 2009. Modifications incorporated in CpG motifs of oligodeoxynucleotides lead to antagonist activity of toll-like receptors 7 and 9. J. Med. Chem. 52, 5108–5114.

Zheng, C., Feng, J., Lu, D., Wang, P., Xing, S., Coll, J.L., Yang, D., Yan, X., 2011. A novel anti-CEACAM5 monoclonal antibody, CC4, suppresses colorectal tumor growth and enhances NK cells-mediated tumor immunity. PLoS One 6, e21146.

Zhou, H., Fuks, A., Alcaraz, G., Bolling, T.J., Stanners, C.P., 1993. Homophilic adhesion between Ig superfamily carcinoembryonic antigen molecules involves double reciprocal bonds. J. Cell. Biol. 122, 951–960.

Zhu, M.Z., Marshall, J., Cole, D., Schlom, J., Tsang, K.Y., 2000. Specific cytolytic T-cell responses to human CEA from patients immunized with recombinant avipox-CEA vaccine. Clin. Cancer Res. 6, 24–33.

Zimmer, R., Thomas, P., 2001. Mutations in the carcinoembryonic antigen gene in colorectal cancer patients: implications on liver metastasis. Cancer Res. 61, 2822–2826.