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 MSS 3M2, Canada
\(^b\)Department of Medical Biophysics, University of Toronto, Toronto, Ontario MSG 2M9, Canada
\(^c\)Physical Sciences, Sunnybrook Research Institute, 2075 Bayview Avenue, Toronto, Ontario M4N3M5, Canada

**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.

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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/anokis 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 MSS 3M2, Canada. Tel.: +1 416 480 5710.
E-mail address: gariepy@sri.utoronto.ca (J. Gariépy).
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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 (A1, B1, A2, B2, A3 and B3) 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 oncoprogens 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., 2001; Kitsuki et al., 1995; Ordonez et al., 2000; Scretton 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 A3B3 domains on distinct tumour cells (defined as homophilic interactions) or its association with extracellular markers (defined as heterophilic interactions) such as its association with s3p1 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 A3B3 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 ACA GAC G- (25N)-CGT ATG CTT CCG TTC GTT GCT C 3′. The forward primer 5′ GAC GAT AGC GGT GAC ACA G G′ 3′ and reverse primer 5′ GAG CGA CGG ACG GAA GGC 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 A3B3, or rCEA N. Briefly, 96-well flat-bottomed Falcon microtiter plates (Becton–Dickinson Biosciences, Franklin Lakes, NJ) were coated with either N or A3B3 domain (1 µg/well in 100 µl) in coating buffer (0.2 M carbonate/bicarbonate, pH 9.4) at 37 ºC. 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 ºC 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 hors eradise 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 Schlim (National Cancer Institute, Bethesda, Maryland). The cervical adenocarcinoma cell line HeLa (ATCC No CCL-2) as well as transfected cell lines HeLaCEACAM1, HeLaCEACAM3, HeLaCEACAM5, HeLaCEACAM6 and HeLaCEACAM9 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 ºC, 5.0% CO2 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 × 10⁴ 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 A3B3 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 (−CaCl2, −MgCl2) and resuspended at a concentration of 10⁶ cells/ml in cold PBS. Aptamers were then added to 1.0 × 10⁶ 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 ºC. 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 × 10⁵ 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 microlipers. Tumour volumes were calculated using the modified formula where the volume of the tumour (mm³) equals [lx² × 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 \times 10^5 cells/well in DMEM medium containing 10% FBS. Aptamers at a concentration of either 250 \mu g/ml (10 \mu M) or 2.5 mg/ml (100 \mu M) were incubated with the cells in medium for 24 h at a volume of 100 \mu l. 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 \mu l; 500 \mu g/ml, 20 \mu M) and cApt (200 \mu l; 500 \mu g/ml, 20 \mu M) as well as a TLR9 ligand CpG ODN (5'-TCCAT-GACGTCTTGCAGTT-3'); type B murine, ODN 1826, Invivogen, CA; (200 \mu l; 50 \mu g/ml, 500 nM) were dissolved in sterile USP saline. These oligonucleotides (200 \mu l) were administered intraperitoneally to 6–8 weeks old C57BL/6 mice. An untreated group of mice received an injection of 200 \mu l of saline alone. Three hours after injection, mice were sacrificed and their serum was collected for analysis. Serum IL-8 and TNF\alpha concentrations were determined using the DuoSet ELISA development kits for mouse CXCL1/KC (murine IL-8, R & D systems Inc.) and murine TNF\alpha (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 \leq 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

![Figure 1](image.png)

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 A1B3 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 A3B3 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 A3B3 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’ GCATGAC 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 impedance-based plates (E-plates) pre-coated with the rCEA N and A3B3 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 A3B3. 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 A3B3 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 A3B3 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 A3B3 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). Apter 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.

Analysis of the CEACAM-expressing HeLa cells as well as the CEA+ MC38.CEA cells, CEA- HeLa and MC38.CEA cells demonstrated that aptamers N54 and N56 specifically bound to MC38.CEA and HeLaCEACAMs 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). Apter N56 binding to MC38.CEA and HeLaCEACAMs resulted in a ~7-fold increase in mean fluorescence signal intensity (Figure 5B and D). Apter N54 binding to MC38.CEA and HeLaCEACAMs 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 (Kd) 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
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 ~5 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 µM; 100 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 Th1 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
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 A3 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 A3 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 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) HeLaCEACAM5. Aptamers N54 and N56 did not detect the presence of other CEACAM family members as shown by low mean fluorescence intensities observed for (E) HeLaCEACAM1, (F) HeLaCEACAM3, (G) HeLaCEACAM6, (H) HeLaCEACAM8 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).
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 Aβ3.

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
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, CEACAM3, 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 CEA- 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 aptamers 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.

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 N54 and N56 in two key aspects. First, this RNA aptamer differs from the present DNA aptamers 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 CEACAM6: 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).

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 TNF-α (Figure 7B and C). As projected, aptamer

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).
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 bind 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.

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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


