Programmable multivalent display of receptor ligands using peptide nucleic acid nanoscaffolds

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Multivalent effects dictate the binding affinity of multiple ligands on one molecular entity to receptors. Integrins are receptors that mediate cell attachment through multivalent binding to peptide sequences within the extracellular matrix, and overexpression promotes the metastasis of some cancers. Multivalent display of integrin antagonists enhances their efficacy, but current scaffolds have limited ranges and precision for the display of ligands. Here we present an approach to studying multivalent effects across wide ranges of ligand number, density, and three-dimensional arrangement. Using l-lysine γ-substituted peptide nucleic acids, the multivalent effects of an integrin antagonist were examined over a range of 1–45 ligands. The optimal construct improves the inhibitory activity of the antagonist by two orders of magnitude against the binding of melanoma cells to the extracellular matrix in both in vitro and in vivo models.
Multivalency describes the simultaneous binding of multiple ligands on one molecular entity to multiple receptors on another entity. Multivalent interactions require precise spatial orientation of ligands and receptors at the nanometer scale, and dysregulation can be responsible for progression of diseases, such as the metastasis of cancer. Integrins are a class of membrane-associated proteins that mediate cell attachment and motility through multivalent binding, and a subset of these proteins (such as αβ₃) bind to the Arg-Gly-Asp (RGD) tripeptide sequence motif of extracellular matrix proteins [1,2]. The expression of integrin αβ₃ is increased on certain tumour cells [3], and antagonists, such as Cilengitide [4], have shown efficacy in clinical trials for metastatic melanoma and glioblastoma [5,6]. Multivalent display of integrin antagonists has been used to enhance their efficacy [7–12], but current scaffolds for the display of ligands have physical limitations that constrict the range and precision of multivalent arrangements that can be explored [13]. Herein, we present an approach to make ligand-conjugated scaffolds that supports comprehensive screening for multivalent effects across wide ranges of ligand number, density and three-dimensional arrangement.

Historically, the development of synthetic scaffolds at the nanometer scale for the multivalent display of ligands can be broken down into two strategies: step-by-step and shotgun. The step-by-step approach involves sequentially attaching individual, ligand-containing units through covalent bonds (Fig. 1a) [14]. In this case, the number of desired ligands dictates the number of required synthetic steps. Although this method yields more precision in terms of ligand number and relative orientation, in most cases, it is logistically impractical for valencies greater than ~10 ligands. In contrast, the shotgun approach involves the single-step coupling (or polymerization) of multiple ligands to a pre-existing scaffold, such as a dendrimer, gold nanoparticle, polymer or protein (Fig. 1b) [15]. Although this can lead to high valencies, it is at the expense of knowing the exact number and/or relative orientation of the ligands. Furthermore, shotgun methods often result in an unknown, complex mixture of species that may further complicate analysis of biological activity. Translation of nanotechnology to medical therapies relies on nanometer-scale scaffolds in which bioactive ligands can be displayed with a high degree of precision to facilitate optimization of biological activity [16,17]. Ideally, the synthesis of such scaffolds would be facile and flexible, allowing for the rapid study of bioactivity over a wide range of different ligand valencies and densities.

Here we present a scaffold and general strategy that overcomes the limitations of previous approaches (Fig. 1c). The core of the scaffold is a hybrid of peptide nucleic acid (PNA) and single-stranded DNA (ssDNA). PNA molecules are oligomers with a peptide-like backbone, typically formed from aminoethylglycine (aeg), with nucleic acid bases as sidechains (Fig. 2a). This allows for stable, complementary binding to DNA or RNA [18,19]. Substituting one or more of the regular aegPNA residues in the synthesis with a monomer that contains an attachment point allows cyclic-RGD-containing ligands, which are antagonists of αβ₃ integrins, to be placed anywhere along the PNA. For this step, we used L-lysine γ-substituted peptide nucleic acid (1κγ-PNA), whose lysine sidechain is available for conjugation and does not interfere with nucleic acid binding [20–22]. Finally, using nucleic acid-driven assembly of ligand-conjugated 1κγ-PNA oligomers and ssDNA containing varying numbers of repeating complementary sequences, we can rapidly form libraries of multivalent molecules with precise numbers and densities of ligands (Figs 1c, 2b,c). For this report, we designed 1κγ-PNA to control the assembly of a derivative of Cilengitide into fifty-two individual multivalent constructs over a range of 1–45 ligands that spanned different ligand arrangements and densities. The biological activities of each construct were examined and the optimal construct improves the inhibitory activity of the drug by two orders of magnitude against the binding of melanoma cells to the extracellular matrix in both in vitro cell-based and in vivo mouse models. We anticipate that scaffolds constructed from 1κγ-PNA will greatly impact studies on multivalent display because the scaffold can be prepared with many different biological ligands and varied ranges of ligand valency, density and arrangement can be explored with high levels of precision for each construct.

**Results**

**Multivalent library.** To demonstrate the utility of our strategy, we developed a library of multivalent PNA–DNA complexes to block the attachment of metastatic melanoma cells to the extracellular matrix. For the ligand, we used a cyclic-RGD analogue, cycloArg-Gly-Asp-dPhe-Lys (c(RGDfK)) [4], with a short polyethylene glycol (PEG) linker to competitively bind to the αβ₃ integrins on the cell’s surface. The c(RGDfK) ligand had previously been examined with...
valencies from 2 to 16 using step-by-step approaches\(^3\), and with average valencies between 13 and 52 with shotgun approaches\(^3,23\).

In contrast, we designed a 52-member library that systematically varies the position, density and number of ligands from 1 to 45 (Fig. 3a). To modulate the positions and density of ligands, we synthesized four different 12-residue PNA oligomers: (A) single ligand at the amino-terminus, (B) single ligand at the centre, (C) two ligands, and (D) three ligands (B, C and D have the ligand attached through a \(\text{Y-Ky-PNA} \) sidechain, Supplementary Fig. S1). Each one of these PNAs was annealed with one of thirteen different ssDNAs with repeats of the complementary sequence from 1 to 15 (Supplementary Table S1). To identify each construct, we refer to a complex consisting of a ssDNA with \(x \) adjacent sequence repeats complementary to a PNA with sidechains as DNA:PNA-\(\text{Y}_x\), where \(x \) is an integer from 1 to 15 and \(Y \) is a letter (A–D) representing one of the four different PNAs.

Cell-based screen for multivalent effects. The first test was a screen to determine the ability of each member of the library to prevent C32 human melanoma cells from attaching to a vitronectin-coated surface (Fig. 3a)\(^24\). The expression and specific function of \(\alpha_v\beta_3\) integrin for attachment has been previously established\(^9\). The first test was a screen to determine the ability of each member of the library to prevent C32 human melanoma cells from attaching to a vitronectin-coated surface (Fig. 3a)\(^24\). The expression and specific function of \(\alpha_v\beta_3\) integrin for attachment has been previously established\(^9\).

The experiments were controlled by the use of unattached c(RGDfK) molecules alone, which allowed results from different days to be normalized. Each experiment was performed at six different concentrations of each DNA:PNA-\(\text{Y}_x\) inhibitor, and each concentration was repeated in triplicate, to determine IC\(_{50}\) values. The results are shown in Figure 3b, Supplementary Figure S2 and Supplementary Table S2. As the inhibitory activities of PNAs A and B were similar, the two sets of results were combined into one series (A/B) in Figure 3b. When increasing the number of ligands per PNA from one to two to three (PNA A/B to C to D), the activity continually increases when each PNA is complexed to complementary DNA with one consensus sequence (IC\(_{50}\) values are 477, 169 and 67 nM, respectively, for DNA:PNA-\(\text{Y}_x\)). This suggests that DNA:PNA-\(\text{Y}_x\) can bind three \(\alpha_v\beta_3\) integrin receptors simultaneously. We confirmed that such a multivalent interaction is sterically possible by developing an atomic-scale computer model of the complex with an arbitrary arrangement of the receptors in a cell membrane (Fig. 4). Increases in activities also occur as the number of consensus sequence repeats in the ssDNA increases. The results show that the activity plateaus at around 5–11 repeats of the consensus sequence in the ssDNA (depending on which PNA is used), with maximal improvement of inhibitory activity approximately two
orders of magnitude greater than that of the c(RGDfK) control. The effect of increasing the distance between adjacent PNAs was examined with PNA-B and ssDNA consisting of complimentary consensus sequences that were separated by extra thymines (Supplementary Fig. S3). Although some improvement in activity was observed using DNA with two and three consensus sequences, the effect was not significant with longer DNAs. We interpret these data to indicate that proper spacing between ligands is important for interaction with multiple integrin receptors, but, at higher valencies, there is a local concentration effect that simultaneously contributes. These results are consistent with other multivalent assemblies of RGD7,25–28. Examination of the full multivalent landscape (Fig. 3b) allowed selection of the construct DNA:PNA-D5 for further binding and in vivo studies. This construct displays 15 cyclo-RGD ligands and was selected because it has the most potent inhibitory activity with the shortest ssDNA sequence.

**Competition assay.** Quantitative measurements of the dissociation constant for DNA:PNA-D5 binding to αvβ3 were conducted using competitive displacement experiments against radiolabeled 125I-echistatin binding to C32 melanoma cells. Echistatin is a 49 residue snake-venom peptide with a single RGD motif that binds strongly to αvβ3 (Kd = 0.3 nM) and other integrins29. The displacement curves for individual tests of DNA:PNA-D5 and the c(RGDfK) control are shown in Figure 5a. Similar to the previous results (Fig. 3b), the multivalent DNA:PNA-D5 has a dissociation constant, two orders of magnitude lower than monovalent c(RGDfK) (Kd values of 0.16 versus 62.9 nM, respectively). The displacement of 125I-echistatin further confirms that the RGD ligands of DNA:PNA-D5 bind to the receptor sites on the integrins.

**In vivo activity.** Next, we investigated the in vivo activity of DNA:PNA-D5 using a mouse melanoma cell line (B16F10). In vitro analysis using the aforementioned cell-adhesion assay showed B16F10 cells responded to the DNA:PNA constructs in a manner consistent with C32 cells (Supplementary Table S3). B16F10 cells injected into the tail vein rapidly metastasize and form tumours in the lungs30. This mouse model was selected over subcutaneous models because the mechanism of Cilengitide is known to inhibit metastasis under these conditions and provides direct evidence of any improvement owing to multivalent effects31,32. Previous studies have demonstrated that lung colonization by B16F10 cells in this experimental metastasis model can be significantly reduced through the use of integrin receptor ligands31,32. Compared with the control group receiving only B16F10 cells, mice dosed with 2 mg of unconjugated c(RGDIK) (2.4 μmol) showed about a 30% reduction in tumour colonies on killing (Fig. 5b,c) after 14 days. However, mice that were treated with 0.1 mg of DNA:PNA-D5 (1.7×10^-3 μmol of complex, or 2.6×10^-2 μmol c(RGDIK), which amounts to 1% of the number of c(RGDIK) molecules relative to the control sample) showed an average reduction of ~50%. Thus, the efficacy of each c(RGDIK) unit appended to the DNA:PNA-D5 scaffold increased by about two orders of magnitude over c(RGDIK) alone. Mice treated with ssDNA or PNA alone, or with DNA:PNA complexes without attached c(RGDIK) ligands, did not exhibit any reduction in lung colonization.

**Stoichiometry and stability.** Finally, analytical ultracentrifugation was used to verify the stoichiometry and stability of the constructs. Although the DNA:PNA duplexes used in these studies are thermodynamically stable, as evidenced by annealing experiments, higher
PolyOrg. PNA oligomer synthesis was carried out on a 5 and downloading the resin with Boc-protected 3,6-dioxaoctanoic acid (mPEG) to further purification except as indicated. The resin (MBHA, 100–200 mesh, 1% divi-mannan) was manipulated to achieve a well-defined peak for the complex across multiple concentrations. The DNA:PNA-B₃₂ construct is not in equilibrium with remaining free PNA. Although PNA oligomers can aggregate in solution reducing the binding efficiency to complementary sequences, there was no evidence of unbound ssDNA.

Discussion

Complexes of DNA with ligand-modified Ky-PNA should be used to overcome the limitations of traditional nanometer-sized scaffolds and accurately control the presentation of biological ligands. Notably, the ability to rapidly produce a systematically varied library over a broad range of valencies and geometries allows for a clear determination of the optimal configuration. In this case, it was relatively simple to identify the multivalent DNA:PNA-D₂ construct with 15 c(RGDfK) ligands to inhibit αvβ₃ integrin binding with activity two orders of magnitude greater than c(RGDfK) alone. This demonstrates remarkable activity. For example, a regioselectively designed construct models were developed from PDB accession code: 1L5G (ref. 11) and 1PDT (ref. 12). Molecular modelling. Atomic-scale, computer models were developed with the QUANTA Modelling Environment software program (Accelrys). The models were illustrated with the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR01081). The extended αvβ₃ integrin model was developed from the crystal structure of a ‘bent’ conformation, as described by Xiong et al. (PDB accession code: 1JY2). The (cRGDFG) moity was positioned analogous to the cyclic-RGD analogue in the subsequent studies. Cyclo-RGD or PNA complex models were developed from PDB accession code: 1PDQ.

Preparation of multivalent constructs. A solution of 5′-kPNA (1.5 μmol), 3,4-dioxythio-3-cyclobutene-1,2-dione (45 μmol, 30 equiv.), triethylamine (90 μmol, 60 equiv.), 500 μmol of anhydrous DMSO and 250 μl of absolute ethanol was mixed for 3 h. The solution was concentrated and the residue was washed with diethyl ether (3×1.0 ml), and dried under vacuum to produce the squaric-acid–conjugated PNA intermediate as a white solid. The above PNA intermediate was added to a solution of Echistatin (12 μmol, 20 equiv., Peptides International), triethylamine (45 μmol, 30 equiv) in 500 μmol of anhydrous DMSO and 250 μl of absolute ethanol. After 18 h of shaking, the solution was concentrated. The residue was purified by high-performance liquid chromatography (19 mm×130 mm Waters C8 reverse-phase column, 12 ml min⁻¹ flow rate, 0.1% aqueous trifluoroacetic acid and acetonitrile) to obtain the final product (57–90% yield over two steps) as a white solid. Cytotoxicity assays were characterized by measuring cell survival with an Agilent 1200 series quadrupole LC/MS electrospray trap (Supplementary Table S4). To make PNA-DNA complexes, a 1× PBS buffer solution of 20 μM DNA (Supplementary Table S1, Integrated DNA Technologies) was combined with the appropriate concentration of PNA conjugate depending on the repeat unit number on the DNA (ultrafast quantification was performed using either a Nanodrop ND 1000, or an Agilent 8453 UV-Vis Spectrophotometer) at 25 °C. The solution was heated to 92 °C and held for 5 min, and then slowly cooled down to 25 °C over a period of 3 h.

Cell culture and cell attachment assay. C32 and B16F10 cells were maintained in RPMI 1640 medium supplemented with 10% FCS, 2 mm l-glutamine, and 1% penicillin-streptomycin in a humidified incubator at 37 °C in an atmosphere containing 5% CO₂. The cell attachment assay was carried out as previously described. Collagen Type I was purchased from Advanced BioMatrix. Vitronectin, bovine serum albumin (BSA), BSA’s solution, and Echistatin were purchased from Sigma. 96-well flat-bottom plates were purchased from NUNC. All other cell culture media and medium supplements were purchased from Gibco. Either collagen Type I or vitronectin diluted in Dulbecco’s PBS (without Ca²⁺, Mg²⁺) was coated onto 96-well flat-bottom plates (NUNC) overnight at 4 °C. After aspiration of the buffer, nonspecific adherence to plastic was blocked by incubation with DPBS containing 1% BSA at room temperature for 30 min. Cells were collected and washed in DPBS and resuspended at 1×10⁶ cells ml⁻¹ in serum-free RPMI1640 containing 1% BSA. Aliquots of cells (50 μl) were added to each well containing 50 μl of the indicated concentrations of c(RGDfK) or PNA construct diluted in serum-free RPMI1640 containing 0.1% BSA. The plate was incubated at 37 °C for 45 min to allow the cells to attach to the plate. After the incubation, medium was discarded and nonadherent cells were removed by washing the plate with DPBS (with Ca²⁺, Mg²⁺). The number of adherent cells was quantified using the previously described colorimetric hexosaminidase assay (ref. 23).

Unlabeled echistatin. Echistatin iodination was carried out by the Chloramine T (N-chloro tosylamide) method. This procedure typically yielded a specific activity of 17.5 μCi μg⁻¹. Competitive binding assays were carried out using a fixed amount of radioactive tracer added to varying concentrations of unlabelled competitor in the presence of 10⁵ C32 cells per tube. Triplicate samples were incubated at 25 °C on a rocking platform. Bound and free ligands were separated by centrifugation through a Nylor M25 oil cushion. Nonspecific binding was assessed using 20-fold excess unlabelled echistatin as a competitor. The success of a PNA-binding experiment was assessed by comparison with a competitive displacement experiment using c(RGDfK) peptide carried out at the same time. The data was non-linearly fit to the 2-ligand, single receptor, heterologous displacement model: 

\[ \frac{B}{B'_0} = \frac{B}{B'_0} \frac{[S]}{K_D + [S]} + \frac{B}{B'_0} \frac{[S]}{K_D + [S]} \]

The model was fit to the observed activity data using the computer program LIGAND (ref. 24).

Animals and tumour metastasis assay. Eight-to-ten week old female C57BL/ 6NCr mice were obtained from Charles River. All animal protocols used in this study were approved by the National Cancer Institute Animal Safety and Use Committee. Mice were maintained on a 12:12 light-dark cycle and provided with food and water ad libitum. Animals were allowed to acclimatize to their cages for 5 days before initiating the experiment. All mice were injected intravenously with 5×10⁶ C32 cells via the tail vein 14 days after surgery. C32 cells were isolated from the flank and resuspended in 5×10⁵ cells ml⁻¹ C32 cells/RGD or PNA complex. The primary amines on the sidechains were deblocked and coupled to mPEG residues in tandem. Purification of PNA oligomers was carried out using an XBridge Prep. BEH 130 C18 5 μm (10 mm×250 mm) column on an Agilent 1100 HPLC. In all cases, 0.05% aqueous trifluoroacetic acid and acetonitrile were used as solvents.
slowly into the lateral tail vein. Fourteen days later the animals were euthanized with CO2 and their lungs were excised and fixed in Bouin's solution. The number of surface melanoma colonies was counted visually.

**Sedimentation studies on the DNA:PNA-B2 complex** Sedimentation velocity experiments were performed on the DNA:PNA-B2 complex prepared with a slight excess of PNA-B. Initial c(s) analyses were consistent with the presence of both a DNA:PNA-B2 complex at 3.20 S (uncorrected) and excess monomeric and dimeric PNA-B. The well-defined peak for the complex observed at all loading concentrations, together with a constant loading ratio of free PNA-B to complex, would suggest that these complexes are not in equilibrium with each other. Consequently, the appropriate c(s) analysis should allow for an estimate of the complex molecular mass.

A two-dimensional c(s) f(fg) model was initially implemented 45. At all concentrations studied, the projected c(s)* distribution shows the presence of the complex at 3.21 ± 0.02 S, corresponding to an x20,0 of 3.31 ± 0.02 S, as well as monomeric and dimeric PNA-B (Fig. 3a). On the basis of the best-fit average f(fg)/f(o) of 1.95 for the complex, a value consistent with the expected asymmetry of the double-stranded complex, an average molecular mass of 46.0 kDa is estimated for the complex.

Although this value is slightly larger than that expected for the 5:1 complex that has a calculated mass of 42.41875 kDa, it supports the 5:1 stoichiometry for the complex. Data were analysed on terms of a hybrid local continuous/global discrete sedimentation velocity analytical ultracentrifugation. Cell spreading and focal adhesion dynamics are regulated by spacing of integrin ligands. Biophys. J. 92, 2964–2974 (2007).

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Author contributions
E.A.E. and D.W. performed the syntheses, characterizations and assemblies of all PNAs and associated complexes, H.F. and H.S. performed cell-based assays and in vivo studies, C.M.M. developed the conjugation procedure to attach ligands to PNAs, R.G. performed the analytical ultracentrifugation, G.M.-M., M.L.P., and D.D.R. performed the echistatin competition assays and provided and maintained all the cell lines, S.R.D. performed all the computer modelling. The manuscript was written by E.A.E., S.R.D. and D.H.A. The initial ideas were conceived by D.H.A.

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