RNA-Aptamers

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Expanding the Concept of Chemically Programmable Antibodies to RNA Aptamers: Chemically Programmed Biotherapeutics**

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Analysis of the catalytic antibody 38C2, which efficiently catalyzes aldol and related reactions through an enamine mechanism, has proven key to development of chemically programmable antibodies (cpAbs).[1] In 38C2 and related antibody aldolases, an exceptionally nucleophilic lysine residue located on the heavy chain variable domain is critical for activity. This lysine residue can be selectively and covalently labeled with 1,3 diketone- or β-lactam-equipped ligands such as small molecules or peptides to reprogram the binding specificity of the antibody (Figure 1).[2] Thus, in contrast to classic monoclonal antibodies that acquire their

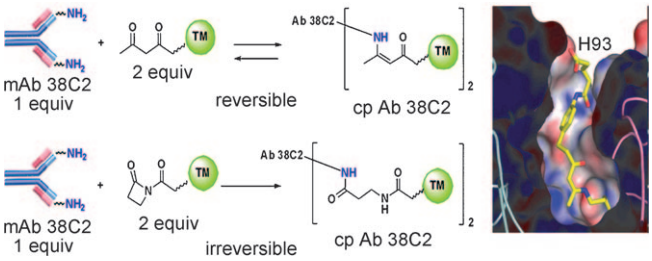

Figure 1. Chemical reactivity of catalytic antibody 38C2 and active site structure showing enamine formation with a unique nucleophilic lysine residue (Lys 93) on the heavy chain of the antibody.[1r] This chemistry can be used to specifically attach targeting molecules (TMs) to the antibody.

specificity through biology (gene rearrangement and hypermutation), cpAbs acquire their specificity through chemistry. Conjugation to the antibody equips the small-molecule or peptide ligand with the pharmacokinetic properties of the antibody and antibody effector functions mediated by the antibody Fc domain. Several studies have shown that this strategy increases the therapeutic efficacy of peptides and small molecules by several orders of magnitude in animal models.[2a,c,f,h] Currently, four peptide-based cpAbs are in human clinical trails (www.clinicaltrials.gov).

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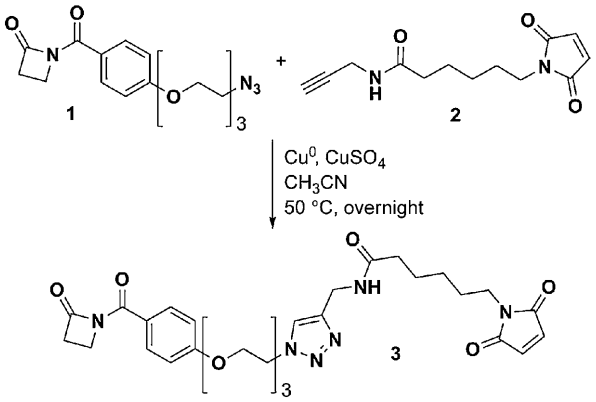
To date, this approach has used small molecules and peptides to direct targeting. However, other classes of therapeutically active molecules, such as aptamers, should benefit from antibody conjugation. Aptamers are structured nucleic acid ligands often selected using the "systematic evolution of ligands by exponential enrichment" (SELEX) procedure.[3] Although aptamers are a promising class of therapeutics because of their excellent binding and inhibitory properties,[4] only a single aptamer, which targets vascular endothelial growth factor (VEGF), is an approved drug.[5]

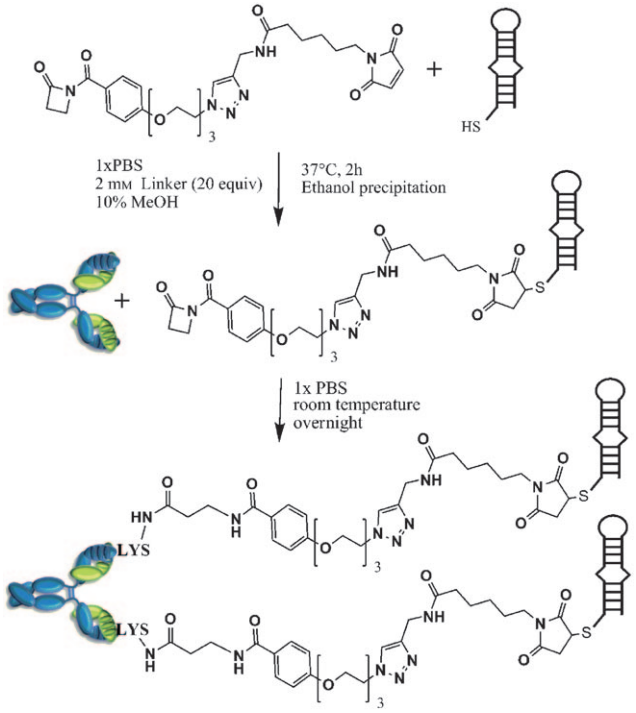
For in vivo applications, aptamers suffer from low chemical stability (these molecules are readily degraded by nucleases in serum)[6] and poor pharmacokinetic properties (circulatory half lives are on the order of several minutes).[7] Nuclease resistance can be enhanced significantly by incorporating 2′ ribose modified nucleobases; 2′-*O*-methyl modified oligonucleotides have acceptable serum stabilities.[8] Other oligonucleotide modifications are also being explored to solve this difficult problem.[9]

To date, most strategies aimed at enhancing the pharmacokinetic properties of aptamers have focused on covalent attachment of ligands such as polyethylene glycol (PEG) to reduce renal clearance.[11] In one study, conjugation of a 40 kD PEG to an aptamer increased the circulatory half-life from several minutes to 23 h.[11d] Data from a phase I clinical trail with PEGylated aptamer ARC1779 indicate that the circulatory half-life is 2 h in humans.[12] The extent and site of PEGylation must be evaluated for each aptamer since not all aptamers tolerate chemical conjugation to PEG molecules above a certain size.[13] Antibody programming provides an attractive alternative to current strategies for extending aptamer half-lifes. By attaching an aptamer to the chemically programmable antibody, the therapeutically valuable binding specificity of the aptamer should be combined with the bivalency, the long in vivo half-life and effector functions of the antibody.

In order to explore the potential of aptamer-based programming of antibodies, we synthesized the β-lactam-based heterobifunctional linker **3** (Scheme 1) with a reactive maleimide moiety for attachment to a thiol-modified aptamer.[2d] In contrast to commercially available linkers like succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), the β-lactam-containing linker **3** leads to site-specific labeling of both variable domains of the antibody as previously shown with other targeting molecules.[2d,e] The synthetic scheme for antibody–aptamer conjugation is outlined in Scheme 2. For our proof of concept experiments, we chose the thiol-modified anti-VEGF aptamer ARC245 since this aptamer is fully 2-*O*-methyl modified, highly nuclease resistant, and its binding and inhibitory properties are well



Scheme 1. Synthesis of heterobifunctional linker **3**.



Scheme 2. Irreversible programming of aldolase antibody 38C2 with aptamer ARC245.

characterized.[11d] Linker **3** was reacted with the aptamer and purified. Mouse cp38C2 and human 38C2 (hucp38C2) were then reacted with the lactam portion to yield an irreversible linkage.

The antibody and ARC245-conjugated antibodies were analyzed by gel electrophoresis as shown in Figure 2. The reactive lysine is located on the heavy chain of 38C2; consistent with this, we observed that only the heavy chain band was shifted to a higher molecular weight under reducing conditions indicative of site-specific labeling as demonstrated previously for other conjugates.[1c,o,r] Labeling was complete as determined by loss of aldolase activity (Supporting Information).

In order to evaluate the binding properties of the ARC245–cpAbs, we performed a previously described dis-

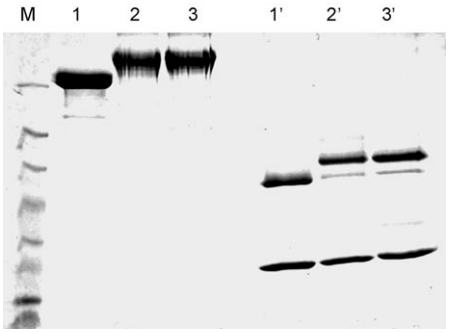


Figure 2. Representative SDS acrylamide gel after conjugation of antibody and aptamer. Lane 1, unmodified 38C2; lane 2, human 38C2 (hu38C2) after conjugation to aptamer; lane 3, mouse 38C2 (38C2) after conjugation to ARC245; lanes 1′, 2′, and 3′, samples as in first three lanes under reducing conditions.

placement ELISA.[14] In this assay, either the unlabeled ARC245 or hucp38C2-ARC245 competed for binding to surface-coated VEGF165 with biotinylated ARC245. Data from this assay is shown in Figure 3. hucp38C2–ARC245 showed a 60-fold lower EC_{50} value than the aptamer alone (0.69 nM vs. 41 nM). The affinities of aptamer and aptamer–

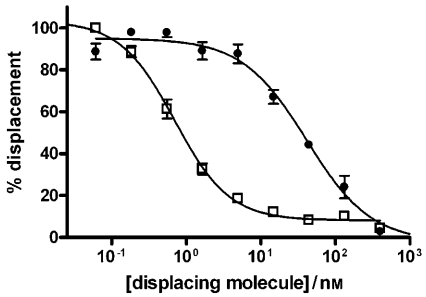


Figure 3. Competitive ELISA with increasing amounts of either unmodified ARC245 (●) or hucp38C2–ARC245 (□) incubated with 20 nM biotinylated ARC245. Biotinylated ARC245 was detected with streptavidin–horseradish peroxidase and percent displacement was plotted.

cpAb were determined by the method of Oroz et al.[14] In this experiment, the affinity of ARC245 was determined to be 1.8 nM and the aptamer–cpAb had a 30-fold higher affinity of 66 pM (Supporting Information). This result shows that the effective affinity of the hucp38C2–ARC245 conjugate was increased significantly relative to the monovalent aptamer ARC245. Other reports indicate that bivalent aptamers have a higher functional affinity than their monovalent counterparts.[15]

The therapeutic target of ARC245 is VEGF, a pro-angiogenic factor excreted by many tumors that stimulates blood vessel growth, helping to maintain a supply of oxygen and nutrients to rapidly dividing tumor cells.[16]

ARC245 is a potent anti-VEGF antagonist that prevents VEGF from binding to the VEGF receptor.[11d] To study the biological activity of cp38C2–ARC245, we performed cell migration assays using human umbilical cord endothelial cells

(HUVEC) in a transwell assay format. Cells were seeded into the top chamber of 8 μm transwell plates and allowed to migrate at 37 °C for 4 h towards the bottom chamber to which 10 ng mL^{-1} VEGF was added.

As shown in Figure 4, addition of 50 nM cp38C2–ARC245 conjugate potently inhibited VEGF-mediated cell migration, demonstrating that the biological activity of ARC245 was retained after antibody conjugation. It is well established that compounds that inhibit VEGF-mediated cell migration in a transwell assay also block proangiogenic signaling in vivo provided they demonstrate favorable pharmacokinetic properties.[17]

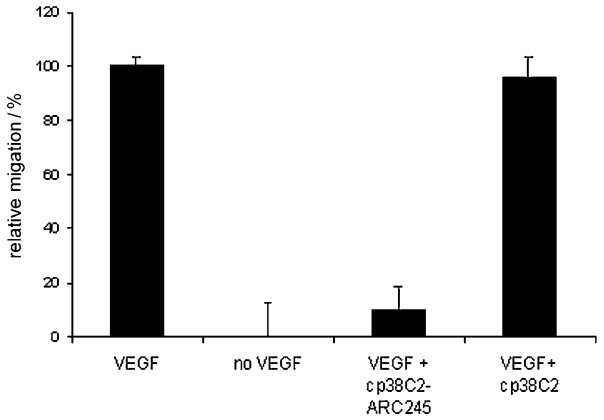


Figure 4. Transwell migration assay of HUVEC cells migrating toward 10 ng mL^{-1} recombinant human VEGF165. cp38C2–ARC245 was applied at a concentration of 50 nM. Cells were allowed to migrate for 4 h; $n=3$, $p=0.014$.

The pharmacokinetic properties of hucp38C2–ARC245 were evaluated in athymic nude mice. hucp38C2–ARC245 (100 μg; 5 mg kg^{-1}) was injected intravenously and blood was collected at various time points up to 96 h post injection via the tail vein. In order to detect the intact antibody–aptamer conjugate an ELISA assay was developed in which hucp38C2 was first captured from serum, and then a biotinylated antisense oligonucleotide was added that annealed to the aptamer. Finally this oligonucleotide was detected with streptavidin–horseradish peroxidase. Based on this analysis, which detected the aptamer, the hucp38C2–ARC245 conjugate showed a clearance half-life of approximately 21 h. In parallel, the concentration of the antibody hucp38C2 was determined from the same blood samples (Supporting Information). This half-life was approximately 68 h. The most likely explanations for the difference in half-lives of the antibody and the aptamer are either nuclease digestion of the aptamer portion or cleavage of the heterobifunctional linker. The addition of a 5′-cap to the aptamer and modifications of the heterobifunctional linker might increase the overall stability of the conjugate; however, the increase in circulatory half-life of the antibody conjugate determined here as compared to that of the free aptamer was very significant. Healy et al. reported that the circulatory half-life of the fully 2′-*O*-Me modified ARC159 is less than 30 min following intravenous dosing.[11a] Thus the cpAb approach substantially increased the circulatory half-life of the aptamer; this should translate into less frequent dosing regimens for aptamer drugs.

In summary, we demonstrated for the first time site-specific conjugation of an aptamer to the aldolase antibody 38C2 to produce an aptamer-programmed cpAb. Conjugation of the VEGF-targeting aptamer ARC245 to the well-characterized chemically programmable antibody 38C2 resulted in a biologically active aptamer–antibody conjugate that had significantly increased functional affinity and a much longer circulatory half-life than the free aptamer. The aptamer–cpAb strategy developed here should be readily transferable to other aptamers. Aptamer-based cpAbs of the type developed here represent a promising new class of aptamer immunotherapeutics that combine the favorable characteristics of aptamers with those of antibodies. This approach might also be applicable to chemically programmed vaccines.[18]

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