Cancer Therapy: Preclinical

Specifically Targeting Angiopoietin-2 Inhibits Angiogenesis, Tie2-Expressing Monocyte Infiltration, and Tumor Growth

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Abstract

**Purpose:** Angiopoietin-1 (Ang1) plays a key role in maintaining stable vasculature, whereas in a tumor Ang2 antagonizes Ang1’s function and promotes the initiation of the angiogenic switch. Specifically targeting Ang2 is a promising anticancer strategy. Here we describe the development and characterization of a new class of biotherapeutics referred to as CovX-Bodies, which are created by chemical fusion of a peptide and a carrier antibody scaffold.

**Experimental Design:** Various linker tethering sites on peptides were examined for their effect on CovX-Body in vitro potency and pharmacokinetics. Ang2 CovX-Bodies with low nmol/L IC50s and significantly improved pharmacokinetics were tested in tumor xenograft studies alone or in combination with standard of care agents. Tumor samples were analyzed for target engagement, via Ang2 protein level, CD31-positive tumor vasculature, and Tie2 expressing monocyte penetration.

**Results:** Bivalent Ang2 CovX-Bodies selectively block the Ang2–Tie2 interaction (IC50 < 1 nmol/L) with dramatically improved pharmacokinetics (T½ > 100 hours). Using a staged Colo-205 xenograft model, significant tumor growth inhibition (TGI) was observed (40%–63%, P < 0.01). Ang2 protein levels were reduced by approximately 50% inside tumors (P < 0.01), whereas tumor microvessel density (P < 0.01) and intratumor proangiogenic Tie2+CD11b+ cells (P < 0.05) were significantly reduced. When combined with sunitinib, sorafenib, bevacizumab, irinotecan, or docetaxel, Ang2 CovX-Bodies produced even greater efficacy (~80% TGI, P < 0.01).

**Conclusion:** CovX-Bodies provide an elegant solution to overcome the pharmacokinetic–pharmacodynamic problems of peptides. Long-acting Ang2 specific CovX-Bodies will be useful as single agents and in combination with standard-of-care agents. Clin Cancer Res; 17(5); 1001–11. ©2011 AACR.

Introduction

Angiogenesis is one of the hallmarks of tumor progression, allowing the tumor to expand beyond the limit of oxygen and nutrient perfusion and eventually metastasize to distant organs (1, 2). Antiangiogenic agents such as bevacizumab, sunitinib, and sorafenib that target VEGF signaling pathways have demonstrated clinical utility in treating solid tumors. However, these agents result in only transient clinical responses and tumors eventually develop resistance and escape the angiogenic blockade. The various mechanisms of resistance can be attributed to the inherent heterogeneity of tumor cells. There is a strong interest in targeting other proangiogenic factors along with VEGF as simultaneous targeting of multiple pathways could translate into more robust therapeutic outcomes. The angiopoietin–Tie system presents an attractive therapeutic target as it plays an important role in tumor angiogenesis and provides a critical link between angiogenic and inflammatory pathways (3).

The human angiopoietin–Tie system consists of 2 type I tyrosine kinase receptors (Tie1 and Tie2) and 3 secreted ligands (Ang1, Ang2, and Ang4; refs. 3, 4). Of the 3 angiopoietin ligands, Ang1 is constitutively expressed in many organs, whereas Ang2 is predominantly expressed in tissues undergoing vascular remodeling (5, 6). Ang2 overexpression in many cancers correlates with poor survival and more invasive cancers (3, 7). No clear correlation was found between Ang1 expression and prognosis in several solid tumors, such as colorectal carcinoma (8), bladder cancer (9), or non–small-cell lung cancer (NSCLC; ref. 10). Recently, in head and neck squamous cell carcinoma, a high level of Ang1 was reported to correlate with better prognosis (11). In a remodeling endothelium, such as those in tumors and diabetic retinopathy, Ang2 acts as an Ang1 antagonist, promotes the dissociation of pericytes,
resulting in unstable vasculature (4). In addition to angiogenesis, Ang1 and Ang2 play a role in the inflammatory and metastatic pathways through their ability to modulate the endothelial barrier and the integrity of the blood vessels (3). High levels of Ang2 induce leaky vasculatures that facilitate the extravasation of lymphocytes and promote the adhesion of rolling leukocytes to blood vessels (12). Circulating Tie2+ monocytes have been shown to contribute to tumor angiogenesis (13–16). Tumors with overexpressed Ang2 are more vascularized and have more Tie2-expressing monocyte penetration (14). These findings suggest that increased Ang2 over Ang1 promotes tumorigenesis and blocking Ang2 is an attractive strategy for cancer treatments. A selective Ang2 trap significantly inhibits tumor growth, whereas treatment with a selective Ang1 trap has no effect on tumor growth in a Colo-205 xenograft model (17). The peptide-body AMG-386 in various clinical trials traps both Ang1 and Ang2 and inhibits tumor growth preclinically (18, 19). Herein we describe the development and characterization of peptide-antibody fusion proteins referred to as CovX-Bodies, for selectively targeting Ang2 as a potential antiangiogenic anticancer agent.

Materials and Methods

Synthesis of peptide phamacophore with the azetidinone linker and preparation of CovX-Bodies

All peptides were synthesized on a Symphony peptide synthesizer, using Rink Amide resin (Nova-Biochem; 100–200 mesh). Briefly, peptides were made using sequential deprotection and coupling with Fmoc-protected amino acids. During the peptide synthesis, e-Mtt-Fmoc-Lys-OH was used at the desired linker tethering site. Once the peptide sequence was assembled on the resin, the ε-amino group of Lys was unmasked by treatment with 1% TFA in dichloromethane, and coupled to the azetidinone (AZD) linker. The tethered peptide was cleaved off the resin by brief exposure to a solution of 95% TFA in water and purified by preparative reverse phase HPLC using a Zorbax SB-C18 column. The purity of the final products was assessed by analytical reverse-phase LC/MS.

Peptide phamacophores with AZD linkers were combined at a 3:1 molar ratio with CVX-200, a humanized IgG1 monoclonal aldolase antibody, used at 20 mg/mL in a buffer of 10 mmol/L histidine, 10 mmol/L glycine, 2% sucrose, pH 6.5. The AZD linker was designed to be recognized and covalently bound only by the reactive lysine in the binding site of CVX-200. After incubating at room temperature overnight, free pharmacophore was removed from the reaction solution by size exclusion chromatography (SEC), using a Superdex 200 10/300 GL column on an AKTA Purifier (GE healthcare). The SEC buffer was 0.5 mol/L sodium chloride, 0.1 mol/L sodium phosphate, pH 6.5. CovX-Bodies were exchanged into PBS using centrifugal filters and their valency was confirmed by LC-MS. This process is referred to as programming hereafter and the resulting peptide-antibody fusions are termed as CovX-Bodies (Fig. 1).

Animal models and dosing agents

For pharmacokinetic studies, male Swiss Webster mice or Sprague Dawley rats (Charles Rivers Laboratories) were used. For rats, jugular vein and carotid arterial cannulae were surgically implanted at Charles River Laboratories and animals were housed separately. CovX-Bodies (10 mg/kg) were injected intravenously through the tail vein for mice.

Figure 1. Generation of CovX-Bodies. A pharmacophore comprising a linker and a bioactive component was synthesized. A spontaneous selective fusion reaction occurs between the AZD ring and the Lys99 of the heavy chain. This nucleophilic reaction transforms the AZD ring into a stable beta alanine-based peptide bond creating CovX-Bodies.
and via implanted jugular vein cannulae for rats. For tumor studies, young adult female 
Nu-Fax™ mice (Charles River Laboratories) were housed 10 per cage in a tempera-
ture- and light-controlled vivarium. Tumor cell lines Colo-205 and MDA-MB-435 were cultured in RPMI 1640 med-
ium with 10% FBS in a 5% CO2 incubator with 98% humidity to subconfluence. A total of $5 \times 10^6$ Colo-205 cells alone or $5 \times 10^6$ MDA-MB-435 cells mixed with Matrigel (1:1; BD Bioscience) were injected subcutaneously into the upper right flank of each mouse. The tumor volume was calculated using the formula: Volume = $(\text{Length} \times \text{Width}^2) / 6$. All animal experiments were conducted after protocol approval by the CovX Institution al Animal Care and Use Committee.

**Ang2-Tie2 competition and pharmacokinetic ELISA**

Competition ELISA was used to determine the in vitro potency of CovX-Bodies in blocking the Ang2-Tie2 inter-
action. Human Tie2-Fc, human Ang2 protein, biotinylated anti-human Ang2 antibody, and streptavidin horseradish 
peroxidase (HRP) were from R&D Systems. Briefly, high-binding half-well plates were coated with 50 ng Tie2-Fc in 
PBS. Compounds were diluted in the presence of 50 ng/mL Ang2. Bound Ang2 was detected by a biotinylated anti-
Ang2 antibody used at 2 $\mu$g/mL. For pharmacokinetic analysis of CovX-Bodies, after intravenous administration of 
CovX-Bodies, blood samples ($\sim 0.08$ mL/bleed) were taken from mice via retro-orbital sinus bleeds into capillary 
blood collection tubes (BD Biosciences) with protease inhibitor cocktail (Sigma) starting at 5 minutes up to 7 or 14 days. The blood samples were allowed to clot on ice for 30 minutes. Samples were then centrifuged at 12,000 rpm for 10 minutes at 4°C to collect serum and immedi-
ately stored at $-80^\circ$C until analysis. The detection assay conditions were optimized for reproducibility and sensi-
tivity (limit of detection was as low as 10 ng/mL). Fifty nanograms of Ang2 protein in PBS was coated as a capture 
reagent overnight at 4°C. The plates were blocked with Superblock (Slytek) for 1 hour at room temperature. Samples and standards were prepared in 10% mouse serum 
in Superblock, added to blocked plates, and incubated for 1 hour. An HRP anti-human IgG (1:2,000; Bethyl Labora-
tories) was used to detect CovX-Bodies. Data analysis was undertaken with WinNonlin version 4.1 (Pharsight Cor-
poration) using a weighted $1/V^2$ 2-compartment model.

**Tumor xenograft studies**

Subcutaneously inoculated tumors staged to the desired volume (average about 200 mm$^3$) were randomized and 
dosed accordingly. Ten animals were used per group. Bev-
acizumab (Avastin), docetaxel (Taxotere), irinotecan (Camp-
tosar), and 5-FU (Fluorouracil) were diluted to the desired dosing concentrations with either PBS (for bevacizumab) or 
5% dextrose in water (for docetaxel, irinotecan, and 5-FU), immediately prior to administration, and were administered intraperitoneally in a volume of 0.2 mL per mouse. CovX-
Bodies were administered intravenously in a volume of 
0.2 mL per mouse. A 16 mg/mL solution of sorafenib 
(Biomol) was prepared fresh every 3 to 4 days in cremo-
phor/ethanol (50:50; Sigma cremophor EL, 95% ethanol). The final dosing solution (4 mg/mL) was prepared by dilut-
ing with water. Sunitinib was prepared in 0.5% polysorbate 
80, 10% polyethylene glycol 300, and 1:1.02 molar ration of 
hydrochloric acid to sunitinib, pH 3.5. Sorafenib and suni-
tinib were dosed by oral gavage. For combination studies, the 
control groups were given vehicles used for both agents. Tumor growth data were analyzed using 2-way ANOVA with 
Bonferroni posttests between groups using Graphpad Prism 
Ver 5.0 and depicted as mean ± SEM.

**Histologic analysis**

For tumor necrotic studies, four 5-µm sections, 5 sections 
part from each other, were prepared and stained with 
hematoyxlin and eosin (H&E) following standard histologic 
procedures. To determine necrotic and viable tumor 
area, whole field images of each stained tumor section were 
acquired at $10 \times$ magnification, using a Qimaging Micro-
publisher 5.0 RTV camera coupled with a Nikon Eclipse 80i 
microscope equipped with an automatic stage. ImagePro 
6.0 was used to quantify the necrotic and viable area by 
using the tracing function and a common macro for all 
images. For CD31-positive vasculature staining, snap-fro-
zen tumor sections were oriented and embedded in OCT 
blocks. Three sections (5 µm) from different depth of the 
tumor mass were used for each tumor. CD31 antibody and 
anti-Rat IgG detection kit (Pharmingen) were used for 
blood vessel staining. CD31-positive area in each tumor 
was imaged using a QImaging Micropublisher 5.0 RTV 
camera coupled with a Nikon Eclipse 80i microscope 
($20 \times$). Five images were taken across each tumor section 
where CD31 staining was greatest in the viable rim so that a 
total of 15 images were taken for each tumor. The number 
of blood vessels was determined by operators blinded to 
the study design and the total number of vessels from the 
15 images was presented as microvessel density (MVD). For 
Tie2+ macrophages, the primary antibodies were mouse 
anti-Tie2 (Pharmingen) and rat anti-CD11b (Abcam). Three images ($40 \times$) from the area with the greatest 
CD11b staining were taken and counted by operator 
blinded to the treatments. Nine to 10 animals were used 
per group and 1 animal from the treatment group was lost 
during the study, which was not related to the treatment 
because all other animals in the group were normal and not 
found in subsequent studies. Histologic data were analyzed 
by 1-way ANOVA followed by Dunnett’s post hoc analysis.

**Results**

**Tethering at different sites of a peptide 
pharmacophore impacts the activity and 
pharmacokinetics of CovX-Bodies**

Fusing peptides to various scaffolds through their N- or 
C-termini has been studied extensively, while our approach 
allows us to fuse peptides to a specially-designed scaffold 
not only through their termini but also via middle residues. 
The AZD linker used here creates an irreversible covalent
bond between a peptide and the scaffold and is different from that used in previously published CovX-Bodies based on a reversible diketone linker. The improvement on pharmacokinetics with these reversible linkers was not sufficient for most clinical drug development (20, 21). A series of CovX-Bodies were made, as shown in Table 1, by moving the tether attachment site across the length of the peptide. This was done by a serial substitution of Lys at each position of the peptide using the ε-amino group of the Lys side chain as a site for tethering the AZD linker (Fig. 1). The resulting CovX-Bodies were tested in an in vitro Ang2-Tie2 competition ELISA assay. The pharmacokinetic profile of the resulting CovX-Bodies, CovX-Bodies linked through either the N- or C-termini were active with IC50s of 1.83 and 0.22 nmol/L, respectively. Tethering at certain internal residues (4, 6, 7, 10, and 13) resulted in a complete loss of antagonist activity (IC50 > 1,000 nmol/L) of CovX-Bodies, whereas tethering at positions 5, 14, and 17 resulted in significantly compromised potency. All other conjugations had IC50s within the range of the terminally linked ones (Table 1). The pharmacokinetic profile of the CovX-Bodies showed a dependence on tether site as molecules bearing a tether at internal sites showed better half-lives compared with the ones tethered near either terminus. Thus, CovX-Bodies with internal tethers (9, 11, 16, and 18) resulted in much improved in vivo stability with a beta half-life of 72 to 94 hours, when compared with terminal fusions, with a beta half-life of about 20 hours (Table 1).

### Table 1. The in vitro potency of CovX-Bodies tethered at different residues, indicated by K(0P), in blocking Ang2–Tie2 interaction and their mouse pharmacokinetics

<table>
<thead>
<tr>
<th>Compound#</th>
<th>Sequence</th>
<th>IC50, nmol/L</th>
<th>T1/2, h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide 1</td>
<td>QK(Ac)YQPLDELDK(Ac)TLYDFMLQGG</td>
<td>98</td>
<td>0.1</td>
</tr>
<tr>
<td>1</td>
<td>K(0P)K(Ac)YQPLDELDK(Ac)TLYDFMLQGG</td>
<td>1.8</td>
<td>24</td>
</tr>
<tr>
<td>2</td>
<td>K(0P)YQPLDELDK(Ac)TLYDFMLQGG</td>
<td>0.3</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td>K(0P)K(0P)QPLDELDK(Ac)TLYDFMLQGG</td>
<td>0.2</td>
<td>18</td>
</tr>
<tr>
<td>4</td>
<td>K(0P)YK(0P)QPLDELDK(Ac)TLYDFMLQGG</td>
<td>&gt;1,000</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>K(0P)YQK(0P)QPLDELDK(Ac)TLYDFMLQGG</td>
<td>44.2</td>
<td>ND</td>
</tr>
<tr>
<td>6</td>
<td>K(0P)YQPK(0P)QPLDELDK(Ac)TLYDFMLQGG</td>
<td>&gt;1,000</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
<td>K(0P)YQPLK(0P)QPLDELDK(Ac)TLYDFMLQGG</td>
<td>0.3</td>
<td>35</td>
</tr>
<tr>
<td>8</td>
<td>K(0P)YQPLDK(0P)QPLDELDK(Ac)TLYDFMLQGG</td>
<td>2.0</td>
<td>72</td>
</tr>
<tr>
<td>9</td>
<td>K(0P)YQPLDELDK(0P)QPLDELDK(Ac)TLYDFMLQGG</td>
<td>&gt;1,000</td>
<td>ND</td>
</tr>
<tr>
<td>10</td>
<td>K(0P)YQPLDELDK(0P)K(Ac)TLYDFMLQGG</td>
<td>0.3</td>
<td>74</td>
</tr>
<tr>
<td>11</td>
<td>K(0P)YQPLDELDK(0P)TK(0P)LYDFMLQGG</td>
<td>0.1</td>
<td>56</td>
</tr>
<tr>
<td>12</td>
<td>K(0P)YQPLDELDK(0P)TLK(0P)LYDFMLQGG</td>
<td>&gt;1,000</td>
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<tr>
<td>13</td>
<td>K(0P)YQPLDELDK(0P)TLK(0P)LYDFMLQGG</td>
<td>32.8</td>
<td>ND</td>
</tr>
<tr>
<td>14</td>
<td>K(0P)YQPLDELDK(0P)TLK(0P)LYDFMLQGG</td>
<td>0.2</td>
<td>66</td>
</tr>
<tr>
<td>15</td>
<td>K(0P)YQPLDELDK(0P)LYDFMLQGG</td>
<td>0.3</td>
<td>94</td>
</tr>
<tr>
<td>16</td>
<td>K(0P)YQPLDELDK(0P)LYDFMLQGG</td>
<td>19.5</td>
<td>ND</td>
</tr>
<tr>
<td>17</td>
<td>K(0P)YQPLDELDK(0P)LYDFMLQGG</td>
<td>0.6</td>
<td>72</td>
</tr>
<tr>
<td>18</td>
<td>K(0P)YQPLDELDK(0P)LYDFMLQGG</td>
<td>0.1</td>
<td>65</td>
</tr>
<tr>
<td>19</td>
<td>K(0P)YQPLDELDK(0P)LYDFMLQGG</td>
<td>0.1</td>
<td>35</td>
</tr>
<tr>
<td>20</td>
<td>K(0P)YQPLDELDK(0P)LYDFMLQGG</td>
<td>0.3</td>
<td>27</td>
</tr>
<tr>
<td>21</td>
<td>K(0P)YQPLDELDK(0P)LYDFMLQGG</td>
<td>0.2</td>
<td>20</td>
</tr>
<tr>
<td>22</td>
<td>CVX-060</td>
<td>QK(Ac)YQPLDELDK(0P)TYLDQFMLQGG</td>
<td>0.5</td>
</tr>
<tr>
<td>CVX-060T</td>
<td>K(0P)YQPLDELDK(0P)TYLDQFMLQGG</td>
<td>342</td>
<td>ND</td>
</tr>
<tr>
<td>CVX-87</td>
<td>K(0P)YQPLDELDK(0P)TLYDFMLQGG</td>
<td>0.2</td>
<td>61</td>
</tr>
<tr>
<td>Peptide 2</td>
<td>TNFMPMDDLEK(0P)RLYEOFILQGG</td>
<td>5.4</td>
<td>ND</td>
</tr>
<tr>
<td>CVX-37</td>
<td>(DFB)TNFMPMDDLEK(0P)RLYEOFILQGG</td>
<td>0.4</td>
<td>26</td>
</tr>
<tr>
<td>CVX-51</td>
<td>(0P)TNFMPMDDLEK(0P)RLYEOFILQGG</td>
<td>1.8</td>
<td>28</td>
</tr>
</tbody>
</table>

NOTE: Compounds 1–10, 12–22, CVX-32, CVX-060, CVX-87, CVX-37, and CVX-51 were all tested as CovX-Bodies, while peptides 1, 2, and CVX-060T (the pharmacophores of CVX-060) were also tested as controls. The IC50s were average data from at least 3 independent preparations for each compound.

Abbreviations: ND, not determined; 0P, no PEG unit between peptide and the AZD linker; K(Ac), acetylated lysine; DFB, 1,5-difluorobenzoyl.
However, with different peptide pharmacophores (CVX-37 and CVX-51), no significant improvement in pharmacokinetics was observed by linking through the middle residue (Table 1). For compound CVX-32, changing the linker by modifying the PEG length or the linking amino acid to diamino butyric acid (Dab) or diamino propionic acid (Dap) did not have an effect on the potency or pharmacokinetic profile (data not shown).

**Additional changes in the pharmacophore further improved in vivo stability**

In an attempt to improve its potency and pharmacokinetic profile, CVX-32 was subjected to a series of point mutations with both natural and unnatural amino acids including several nonnatural capping groups at the N-terminus. Most of the changes introduced failed to identify significantly better molecules except when the Lys at position 9 was changed to acetyl Lys, the resulting molecule CVX-060 had a half-life of 110 hours (Table 1). In rats, CVX-060 had a beta half-life of 80 hours, which was improved over CVX-32 and CVX-87 with beta halves of 51 and 55 hours in rats, respectively (P < 0.01). CVX-060 had no direct binding to human Ang3 or its mouse orthologue Ang3 protein coated on plates whereas Tie2 did bind to them (Supplementary Fig. 1A and B). CVX-060 interacted with mouse Ang2 with EC50 of 0.35 nmol/L, compared with 0.04 nmol/L for human Ang2 (Supplementary Fig. 1C and D). Ang1–Tie2 interaction was not affected by CVX-060 up to 1 μmol/L (Supplementary Fig. 2A). CVX-2000, the antibody scaffold, had no effect on the Ang2–Tie2 interaction up to 5,000 nmol/L (Supplementary Fig. 2C). CVX-060 block mouse Ang2 and mouse Tie2 interaction with an IC50 of about 8 nmol/L (Supplementary Fig. 2D).

**CovX-Body programming improved the bioactivity of the peptide pharmacophores**

Ang2 CovX-Bodies efficiently blocked the Ang2–Tie2 interaction in the competition ELISA. When compared with the activity of tethered peptide pharmacophores, programming significantly improved in vitro potency (Table 1, Supplementary Fig. 2B), which may be due to the bivalent nature of CovX-Bodies, resulting in increased avidity of the pharmacophores. The 2 binding sites were demonstrated by surface plasma resonance for CVX-060 and its AZD tethered pharmacophore. The predominant binding site of CVX-060 for Ang2 surface had a KD of 1.8 μmol/L, which corresponded well to the KD of the pharmacophore itself at 4.0 μmol/L. The higher affinity site had a KD of 4.9 nmol/L, which was the affinity of the bivalent complex (Supplementary Fig. 3). With these data, we have demonstrated that we can significantly improve the in vitro stability and bioactivity of peptides.

**Ang2 CovX-Bodies reduced the level of Ang2 protein in tumor mass and inhibited the growth of staged xenografts**

The antitumor activity of the Ang2 CovX-Bodies was evaluated in several staged xenograft models. Tumors were allowed to establish to an average volume of 200 mm3 prior to initiation of therapy. CVX-32, CVX-37, CVX-060, and CVX-87 inhibited the growth of staged Colo-205 tumors significantly (P < 0.01), with TGI values ranging from 40% to 63% (Fig. 2A). When the AZD-tethered peptide pharmacophore CVX-32T of CVX-32 (0.4 mg/kg) was given at the molar equivalent of pharmacophore present in 10 mg/kg CVX-32, it had no effect on the tumor growth demonstrating the benefit of the extended pharmacokinetics of CovX-Bodies (Fig. 2B).

To explore if Ang2 CovX-Bodies reached the target Ang2 protein inside the tumor mass, snap-frozen tumors were homogenized and the total human Ang2 level in these tumors were determined by ELISA. CVX-37 and CVX-060 significantly reduced Ang2 protein level by about 50% in the tumor mass (P < 0.01, Fig. 2C). This was also confirmed by immunohistochemistry and no significant change in Ang1 protein level was found by either ELISA or IHC (immunohistochemistry; data not shown). Although Ang2 is reported to be mostly secreted by endothelial cells, our data and recent reports from other groups indicate that some tumor cells express high levels of Ang2 protein (22–24). In addition, we examined CVX-32 dosed intravenously at 15 mg/kg, twice weekly, in staged A549, HT-29, and A431 models that did not express significant amounts of human Ang2 by ELISA or IHC, we observed 17% to 35% TGI (data not shown), suggesting that Ang2 CovX-Bodies could block mouse endothelial derived Ang2 and caused tumor growth inhibition.

**Tumor vasculature and viability were reduced in treated tumors**

Reducing Ang2 from the tumor mass may affect the formation of new blood vessels. Frozen tumor sections were stained for CD31, an endothelial marker. CD31-positive microvessels were significantly reduced from 40% to 63% (Fig. 2A). When the AZD-tethered peptide pharmacophore CVX-32T of CVX-32 (0.4 mg/kg) was given at the molar equivalent of pharmacophore present in 10 mg/kg CVX-32, it had no effect on the tumor growth demonstrating the benefit of the extended pharmacokinetics of CovX-Bodies (Fig. 2B).

**Tie2+CD11b+ macrophages were reduced in Ang2 CovX-Body–treated tumors**

Circulating Tie2+ monocytes have been reported to contribute to tumor angiogenesis (13–16). We explored the effect of Ang2 reduction on Tie2+ macrophages in tumors by staining for Tie2+CD14+ or Tie2+CD11b+ cells. CVX-37 and CVX-060 significantly reduced the number of Tie2+CD11b+ cells inside the tumors (Fig. 3B and D, P < 0.001) and similar data were observed when CD14 was assessed (data not shown). These data indicate that Ang2 may be responsible for recruiting Tie2+ monocytes into tumors and further contributing to tumor...
angiogenesis in addition to its direct role on endothelial cells.

**Combining Ang2 CovX-Bodies with sunitinib, sorafenib, and bevacizumab significantly inhibited tumor growth**

Patients with high expression of both VEGF and Ang2 have much worse prognosis than those with either factor alone, suggesting that targeting both of them may help these patients (25, 26). Ang2 compromises anti-VEGFR2 vessel normalization effect by increasing vessel permeability (27). However, Ang2 could have both pro- and anti-tumor activities (3). Blocking VEGF function is also known to affect Ang2 expression. In advanced renal cell carcinoma patients treated with sunitinib, plasma Ang2 levels were initially decreased and later increased when patients developed resistance to sunitinib (28). We examined the potential combination benefit of blocking both Ang2 and VEGF pathways by combining CovX-Bodies with known VEGF pathway inhibitors, sunitinib, sorafenib, and bevacizumab, in Colo-205 and MDA-MB-435 xenograft models. CVX-060 was dosed at 10 mg/kg weekly, sunitinib (Fig. 4A) or sorafenib (Fig. 4B) was dosed at 20 mg/kg daily, and bevacizumab (Fig. 4C–E) was given at 3 or 10 mg/kg, alone or in combination. All the agents when dosed alone demonstrated significant tumor growth inhibition. When they were combined with CVX-060, there was a further reduction in tumor growth reflecting additive combination benefits (Fig. 4). For the MDA-MB-435 model (Fig. 4E), the study was designed to determine the delay of tumors.
reaching a predetermined volume (2,000 mm$^3$). The tumors in the combination group did not reach the predetermined limit after 3 months, whereas all the other groups reached the limit approximately 2 months after dosing. These data demonstrate a clear combination benefit of blocking the 2 angiogenic factors.

Combining Ang2 CovX-Bodies with docetaxel and irinotecan significantly inhibited tumor growth

Antiangiogenic treatments could potentially improve the delivery of chemotherapy agents through blood vessel normalization. As demonstrated previously, blocking Ang2 reduced the microvessel density in tumors and its effect on chemotherapy agents was further explored. CVX-060 (10 mg/kg) was combined with 5-FU, docetaxel, or irinotecan. All the agents when dosed alone reduced tumor growth (Fig. 5). When the chemotherapies were combined with CVX-060, there was a further reduction in tumor volume, reflecting an almost complete cessation of tumor growth for combinations with docetaxel or irinotecan. The groups were terminated when the tumor volume reached a predetermined volume (2,000 mm$^3$) and the combination groups of docetaxel and irinotecan never reached this limit during the time of the study. These data indicate that Ang2-specific agents could be combined with standard-of-care chemotherapy agents with potential benefit for patients.
Discussion

Peptides hold significant therapeutic potential, given the readily accessible diversity in their structural and biological properties. However, the promise of peptides remains to be translated into available therapies primarily because peptides are handicapped by their poor in vivo stability. Carrier proteins such as Fc domains of an immunoglobulin have been used as scaffolds to improve the metabolic stability. These constructs are often expressed genetically and are limited to fusing through the N- or the C-terminus of the peptide. Recombinant approaches are also unable to easily incorporate nonnatural structural elements, such as end-capping, amino acid surrogates, and amide bond isosteres, which can often improve the potency and metabolic stability of peptidic pharmacophores. Peptides can also be fused to higher molecular weight biopolymers or coupled to synthetic carriers such as PEG, HES, polysialic acid, which do not have defined stoichiometry. In contrast, with CovX-Bodies, a systematic SAR can be built around tethering site and linker composition for creating the optimal fusion proteins, and the regiochemistry and stoichiometry of the peptide attachment is precisely defined. The peptide pharmacophores can carry a desired combination of natural and nonnatural structural elements. CovX-Bodies provide an elegant solution to overcome the pharmacokinetic–pharmacodynamic limitations of peptidic pharmacophores. The antibody carrier scaffold prevents the peptides

Figure 4. Ang2 CovX-Body CVX-060 inhibited tumor growth when combined with other antiangiogenic agents. Colo-205 tumors were staged to about 200 mm³. Tumors were then treated with weekly intravenous CVX-060 (10 mg/kg), sunitinib (A, 20 mg/kg), sorafenib (B, 20 mg/kg) or combination. Combining CVX-060 and anti-VEGF antibody resulted in significant tumor growth inhibition with Colo-205 (C, 3 mg/kg; D, 10 mg/kg) and MDA-MB-435 xenografts (E). Bevacizumab was administered by weekly intraperitoneal injections. *, P < 0.05 versus vehicle; **, P < 0.01 versus vehicle, P < 0.05 versus monotherapy groups, 2-way ANOVA with Bonferroni test. n = 10 per group.
from being rapidly cleared via renal filtration and also partially protects the peptide from rapid proteolytic degradation. CovX-Bodies are also unique in the respect that the scaffold antibody has no known targets in vivo and can be employed across multiple therapeutic programs. The pharmacophore determines the targeting properties of CovX-Body, whereas the antibody scaffold provides bivalency and long circulating half-life, resulting in an improved dosing regimen relative to the peptide alone. The application of this technology was successfully demonstrated by generating long-acting bioactive CovX-Bodies targeting Ang2, an important target in tumor angiogenesis.

For angiogenesis to occur, preexisting blood vessels are stimulated by several hypoxia-driven angiogenic factors. VEGF and angiopoietins have been increasingly shown to play key roles in this angiogenic switch process. Ang1 is constitutively expressed in normal tissues and is deemed essential to the recruitment of pericytes and maturation of newly formed blood vessels. Ang2 is a key player in the destabilization of preexisting blood vessel through antagonizing the function of Ang1. Ang1 activates Tie2 and results in pericyte recruitment, antipermeability, and anti-inflammatory effects on endothelial cells (29, 30). Conversely, the actions of Ang2 are context dependent. Ang2 acts as a partial Tie2 agonist under certain experimental conditions, such as in the absence of Ang1 (31), when used as a designed pentameric COMP-Ang2 variant (32), or when present in high concentrations (33). A high Ang2/Ang1 ratio correlates with poor prognosis in many solid tumors (7). Conditional overexpression of Ang2 in endothelial cells in vivo confirmed that Ang2 can inhibit Tie2 phosphorylation (34). On the other hand, ectopic overexpression of Ang2 in tumor cells inhibits glioma tumor growth (35, 36), which may be caused by defective angiogenesis due to the death of Ang2-induced unstable blood vessels in the absence of abundant VEGF. These data are consistent with the hypothesis that Ang2 initiates the angiogenic process by promoting the dissociation of pericytes and results in unstable blood vessels. Our data here indicate that systemic and specific reduction of the Ang2 protein can inhibit angiogenesis and tumor growth, supporting Ang2’s role in the pathologic angiogenic initiation process.
In addition to all the angiogenic factors from tumor cells and tumor endothelial cells, other host cells, such as vascular progenitor cells and monocytes, could play critical roles in tumor angiogenesis. Cells that express various monocytic/dendritic cell markers, such as Tie2+ monocytes, among others are involved in tumor vascularization (13). Myeloid cells such as monocytes/macrophages may trigger vessel growth by releasing angiogenic factors and may incorporate directly into nascent blood vessels (37). We demonstrated here that by blocking the Ang2-Tie2 interaction, CovX-Bodies reduced the number of Tie2+ CD11b+ cells inside the tumor mass, indicating that Ang2 may be responsible for the recruitment of Tie2+ monocytes into the tumor mass. Since the identification of angiopoietins, there have been several attempts to interfere with this pathway. Sequestering both Ang1 and Ang2 can inhibit tumor growth, and there were no significant side effects associated with pharmacologic reduction of Ang1 and Ang2 in mice (18). In a phase 1 studies of AMG-386, a peptibody trapping both Ang1 and Ang2, and CVX-060, a CovX-Body selectively trapping Ang2, significant tumor blood flow reduction was observed with dynamic contrast-enhanced-magnetic resonance imaging (DCE-MRI) and early clinical benefit were observed as well (19, 38). The common side effects of these agents are mild and include fatigue, proteinuria, and peripheral edema for AMG-386 (19) and fatigue and proteinuria for CVX-060 (38). If the mild adverse event profile observed in the early clinical trials persists through advanced clinical development, these agents will be very attractive partners in combination with other angiogenic or chemotherapeutic agents. Almost all effective anticancer treatments will eventually fail due to resistance. It has been shown preclinically and clinically that combining targeted treatments, particularly antiangiogenic agents, with chemotherapy agents could potentially improve efficacy. Significant benefit was demonstrated when Ang2 CovX-Bodies were combined with standard of care chemotherapy agents, such as docetaxel and irinotecan, whereas the combination benefit with 5-FU is not as significant. High levels of both VEGF and Ang2 in breast cancer (39), NSCLC (10), ovarian cancer (40), and AML (acute myeloid leukemia; ref. 41) correlate with a worse prognosis than those with either one alone. Targeting Ang2, which was reported to be involved in not only angiogenesis but also in metastasis and inflammation, may help improve the efficacy of anti-VEGF treatments. Significant combination benefit was demonstrated when Ang2 CovX-Bodies were combined with other antiangiogenic agents, such as sunitinib, sorafenib, and bevacizumab. In advanced renal cell carcinoma patients treated with sunitinib, plasma Ang2 levels first decreased and later increased at resistance to the treatment (28). These data suggest that an Ang2 CovX-Body could be a very effective addition to the treatment of solid tumors.

Disclosure of Potential Conflicts of Interest

H. Huang, J. Y. Lai, J. Do, D. Liu, L. Li, J. D. Rosario, V. R. Doppalapudi, S. Frie-Shepherd, N. Levin, G. Woodnutt, R. Lappe, and A. Bhat are employees of CovX Research, Pfizer Inc. C. Bradshaw is a former employee of CovX Research.

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