Chemically programmed antibodies: 
Endothelin receptor targeting CovX-Bodies™

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Abstract—Aryl sulfonamide-based endothelin antagonists were synthesized and covalently linked to the reactive lysine of the m38C2 antibody to create a series of CovX-Bodies. These chemically programmed antibodies behaved as potent endothelin receptor antagonists in vitro and had antitumor efficacy in a prostate cancer xenograft model which, on a molar basis, far exceeded the activity of the parent small molecule.

Keywords: Monoclonal antibodies; Aldolase antibody; CovX-Body™; Endothelin-A; Endothelin-A (ET_A) antagonists; Antitumor efficacy.

The therapeutic potential of antibodies can be expanded by arming them with drugs, toxins, and radionuclides. Although antibodies offer high target specificity, long serum half-life, and Fc-mediated effector functions, development requires years of research and is costly. On the other hand, small molecule or peptide-based leads with excellent activity can be straightforward to find but are often plagued with poor drug-like properties.

In recent publications, Barbas demonstrated that the strengths of certain small synthetic molecules and antibodies could be uniquely combined using an aldolase antibody. In this approach, a reversible, covalent enaminone bond forms in a well-controlled fashion between a β-diketone and active site lysine of the aldolase antibody m38C2. When the β-diketone is incorporated into an extended pharmacophore, the result is a well-characterized, bivalent antibody termed a CovX-Body™ (Fig. 1). This approach effectively combines the function of certain small molecules and peptides with the long serum half-life of an antibody into one unique therapeutic. Barbas discovered a CovX-Body prototype that is based on an integrin targeting pharmacophore. Here, we further expand the technology to include endothelin antagonists, and describe the design and synthesis of endothelin-A (ET_A) antagonist CovX-Bodies. In addition, we also show that ET_A-targeting CovX-Bodies bind ET_A and ET_B receptors and have anti-tumor efficacy in tumor xenograft models.

Endothelins (ET-1, ET-2, and ET-3) constitute a family of vasoconstrictor peptides, produced by the endothelium of blood vessels and many other tissues. Endothelins exert their physiological effects via two specific G-protein coupled receptors termed ET_A and ET_B. Both receptor subtypes are found on smooth muscle cells and mediate vasoconstriction and/or proliferative disorders. The endothelins and their receptors ET_A and ET_B play a major role in tumor growth, proliferation, apoptosis, angiogenesis, and bone metastasis. ET-1 promotes cell growth and suppresses apoptosis of cancer cells. Extensive preclinical and clinical studies with ET_A receptor antagonists (and to a lesser degree ET_B antagonists) have shown potential therapeutic benefits in disease states such as heart failure, pulmonary hypertension atherosclerosis, restenosis, systemic hypertension, various models of cancer, and chronic renal failure.
ETA is up-regulated in several tumor types and ET\textsubscript{A} antagonists are efficacious in prostate tumor models. Atrasentan\textsuperscript{TM}, an ET\textsubscript{A} selective antagonist, recently completed Phase 3 studies for the treatment of prostate cancer.\textsuperscript{10} Of the known ET\textsubscript{A} antagonists, the sulfonamide class is among the most potent and selective.\textsuperscript{11} One such sulfonamide developed by Bristol-Myers Squibb, Edonentan\textsuperscript{TM} (\ref{eq:1}), has completed Phase 3 clinical studies for hypertension. Despite their excellent ET\textsubscript{A} receptor binding, the clinical anti-tumor efficacy of sulfonamide-based ET\textsubscript{A} antagonists has not been reported. In an attempt to define our CovX-Body technology further, we selected ET\textsubscript{A} antagonist \ref{eq:1} as a prototype for an ET\textsubscript{A}-targeting CovX-Body. We sought to demonstrate competitive binding to the target receptor and efficacy in a tumor xenograft model utilizing a CovX-Body equipped with an ET\textsubscript{A} targeting pharmacophore.

**Synthetic strategy and results.** Based upon the published structure–activity relationship\textsuperscript{11} of compound \ref{eq:1}, the 2'-position amide side chain and the oxazole ring attachment site were chosen as sites for attachment of the \(\beta\)-diketone group (Fig. 2). In order to incorporate a diketone functionality at the 2'-position that would allow antibody docking, but not interfere with ET\textsubscript{A} receptor binding, a polyethylene glycol (PEG) spacer was included as shown in compound \ref{eq:2}. Alternatively, the upper oxazole ring was replaced with a PEG spacer-\(\beta\)-diketone functionality resulting in compound \ref{eq:3}. The synthetic strategy for target compound \ref{eq:2} is illustrated in Scheme 1. The

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**Figure 1.** Formation of a CovX-Body.

**Figure 2.** Design of \(\beta\)-diketone containing, ET\textsubscript{A} binding pharmacophores.
convergent synthesis required three key intermediates 4, 5, and 6.

The masked diketone segment 6 was synthesized from 4-nitrobenzyl bromide as shown in Scheme 2. Treatment of 4-nitrobenzyl bromide with the dianion of 2,4-pentanedione, generated with LDA at −78 °C, provided the β-diketone 7 in 40% yield. The β-diketone in compound 7 was converted to bis-ketal derivative 8 before the reduction of the nitro group (Pd/C/H2) to provide aniline 6. The PEG acid 5 was synthesized by the Jones oxidation of the corresponding alcohol.

After synthesis of the biaryl sulfonamide segment 4 according to a reported procedure,11 the condensation of the PEG acid 5 and compound 4 in the presence of water soluble carbodiimide provided the biaryl-sulfonamide 9 (Scheme 3). Treatment of 9 with 6 N HCl under reflux conditions removed both the methoxyethoxymethyl ether (MEM) and tertiary butyl protecting groups to give 10 in 68% yield. Compound 6 was then coupled to 10 in the presence of (3'-dimethylaminopropyl)carbo-
diimide (EDCI) to provide compound 11. Removal of the ketal-protecting group with a catalytic amount of p-toluenesulfonic acid (p-TSA) in acetone completed the synthesis of 2. Compound 2 (2 equiv) was combined with antibody m38C2 (1 equiv) in phosphate-buffered saline, pH 7.4, at room temperature to provide CovX-Body 12 as represented in Figure 1. Reaction completion was confirmed by monitoring the newly generated enaminone bond spectral absorbance (316 nm) normalized to protein concentration.12 Protein concentration was estimated from the 280 nm absorbance ($A_{280} = 1.4$).

Target compound 3, with a tether replacing the oxazole group, was synthesized as shown in Scheme 4. Mitsunobu reaction of 2-iodo, 5-hydroxy benzaldehyde with PEG alcohol provided compound 13 in 55% yield. Carbon–carbon bond formation between the boronic acid derivative11 and compound 13 proceeded smoothly under Suzuki coupling conditions to provide compound 14. The MEM and tert-butyl protecting groups of 14 were removed with 6 N HCl and the subsequent coupling with the diketone segment 6 resulted in 15.

Reductive amination of aldehyde 15 with methylamine and NaBH(OAc)3 and the subsequent treatment with tert-butyl acetyl bromide at −5 °C in the presence of diisopropylethylamine provided 16. Finally, the β-diketone was unmasked by treating 16 with p-TSA in acetone to complete the synthesis of 3. The diketone compound was transformed into CovX-Body 17 by reacting with antibody m38C2 as described for synthesis of CovX-Body 12 above.

The length of the spacer, the attachment point to the pharmacophore, and the chemical compatibility be-

Scheme 1.

Scheme 2. Synthesis of protected β-diketone (6) and PEG diacid (5).
between pharmacophore functional groups and the linker all play a role in tether selection. An ideal tether will allow reaction of the β-diketone moiety with the antibody m38C2 and will also allow the pharmacophore to interact effectively with the targeted receptor. In order to explore the importance of the spacer length in compounds tethered at the 2′-position, we designed spacer length variants 19 and 22 (Scheme 5). For the synthesis of compound 19, a commercially available PEG diacid (a 32 atom spacer length) was coupled with compound 6 in the presence of EDCI to provide compound 18. The sulfonamide 4 was then coupled to 18 and the subsequent diketone unmasking gave 19. Compound 19 was reacted with antibody m38C2 to provide CovX-Body 20.

Compound 22 was synthesized in a similar fashion and transformed into CovX-Body 23 as shown in Scheme 6.
Based on the published structure-activity relationship of compound 1, compounds 27 and 28 were designed to investigate the effect of a phenyl group near the 20-position on ET<sub>A</sub>/ET<sub>B</sub> selectivity. A Mitsunobu reaction was employed as shown in Scheme 7 to incorporate the phenyl group. Addition of masked diketone 6 to PEG acids 23 and 24 followed by ester hydrolysis provided compounds 25 and 26. The biphenyl segment 4 was then added and ketals were removed to give 27 and 28. Diketone derivatives 27 and 28 were then converted into the CovX-Body 29 and CovX-Body 30, respectively.

In vitro ET<sub>A</sub> and ET<sub>B</sub> receptor binding. The ET<sub>A</sub> and ET<sub>B</sub> receptor binding affinity was measured using labeled ET-1 and CHO cells that express human ET<sub>A</sub>...
or ET\textsubscript{B} receptors\textsuperscript{13,14} (Table 1). In all binding assays, the ET\textsubscript{A} targeting CovX-Bodies, the \(\beta\)-diketone analog, and the parent small molecule compound 1 were evaluated. Compound 2 showed an affinity for ET\textsubscript{A} similar to that of the parent compound 1 (IC\textsubscript{50} 1.6 nM vs 1.0 nM). This result is remarkable considering the presence of a long PEG tether in compound 2 compared to compound 1. The corresponding CovX-Body 12, with an IC\textsubscript{50} of 8.6 nM, showed a marginal loss in the ET\textsubscript{A} binding affinity. On the other hand, the ET\textsubscript{B} affinity of compounds 2 and CovX-Body 12 increased slightly relative to compound 1, with a corresponding alteration to the overall selectivity. In contrast, compound 3 had an ET\textsubscript{A} binding affinity almost 50-fold lower than that of compound 1, indicating the importance of the oxazole ring. Increased tether length (in CovX-Bodies 20 and 23) or addition of a phenyl group (in CovX-Bodies 29 and 30) did not enhance ET\textsubscript{A} binding affinity relative to CovX-Body 12.

**Xenograft studies.** We then evaluated in vivo anti-tumor efficacy of CovX-Body 12 in a nude mouse/human xenograft model. Endothelin antagonists inhibit the PC-3 and OVCAR-5 tumor xenograft growth, but there have been no reports on the anti-tumor effects of the biphenyl sulfonamides. In a prostate cancer xenograft (PC-3) study, a 10 mg/kg once weekly dose of CovX-Body 12 inhibited the tumor volume growth by >45% by day 31, compared to the vehicle control. In comparison, a 2 mg/kg daily dose of compound 1 showed 50% tumor growth inhibition by day 31 (Fig. 3). The individual components of a 20 mg/kg dose of CovX-Body 12, antibody m38C2 alone (20 mg/kg/once a week), and compound 2 alone (0.25 mg/kg/once a week) failed to inhibit the tumor growth. Thus, CovX-Body 12 is highly active at a low concentration while compound 2 alone was ineffective even at dosages \(\sim\)40-fold higher, on a molar basis, than those used for CovX-Body 12.

In conclusion, these studies establish the potential of chemically programmed monoclonal antibodies like CovX-Body 12 as a novel and effective class of immunotherapeutics that combine the merits of traditional small molecule drug design with immunotherapy.

**References and notes**