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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.

Monoclonal antibodies (mAbs) have proven effective as therapeutics across a spectrum of diseases, including cancer, heart disease, infection, and immune disorders, as shown by the growing list of mAbs that have been approved by the US Food and Drug Administration and by the European Medicines Agency.[1] The therapeutic potential of antibodies can be expanded by arming them with drugs, toxins, and radionuclides.[2] 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.[3–5] 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.[3,5] 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.[6] 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.[7] 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.[6–9]

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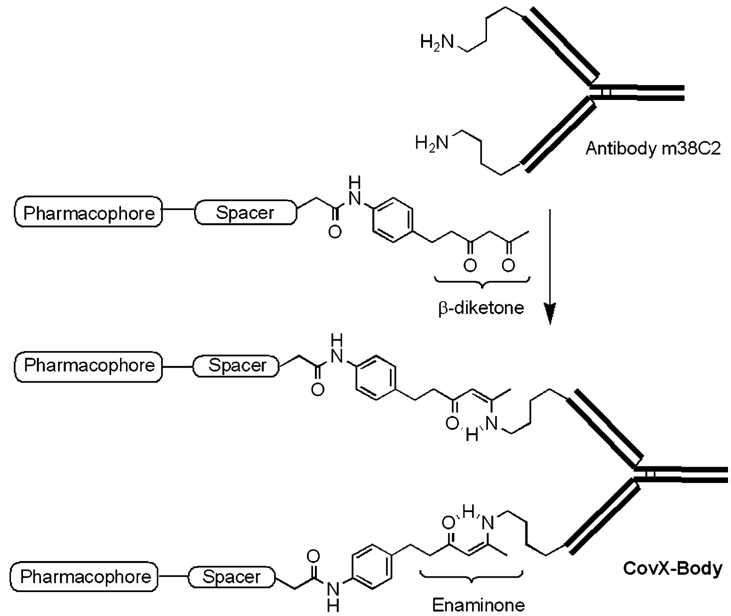


Figure 1. Formation of a CovX-Body.

ET_A is up-regulated in several tumor types and ET_A antagonists are efficacious in prostate tumor models. Atrasentan™, an ET_A selective antagonist, recently completed Phase 3 studies for the treatment of prostate cancer.[10] Of the known ET_A antagonists, the sulfonamide class is among the most potent and selective.[11] One such sulfonamide developed by Bristol-Myers Squibb, Edonentan™ (**1**), has completed Phase 3 clinical studies for hypertension. Despite their excellent ET_A receptor binding, the clinical anti-tumor efficacy of sulfonamide-based ET_A antagonists has not been reported. In an attempt to define our CovX-Body technology further, we selected ET_A antagonist **1** as a prototype for an ET_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_A targeting pharmacophore.

Synthetic strategy and results. Based upon the published structure–activity relationship[11] of compound **1**, the 2′-position amide side chain and the oxazole ring attachment site were chosen as sites for attachment of the β-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_A receptor binding, a polyethylene glycol (PEG) spacer was included as shown in compound **2**. Alternatively, the upper oxazole ring was replaced with a PEG spacer-β-diketone functionality resulting in compound **3**. The synthetic strategy for target compound **2** is illustrated in Scheme 1. The

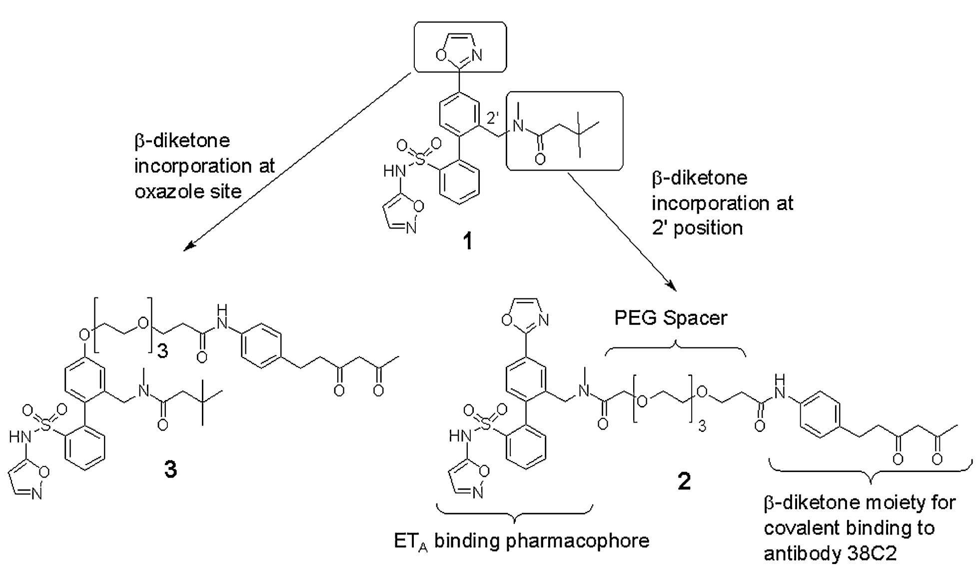
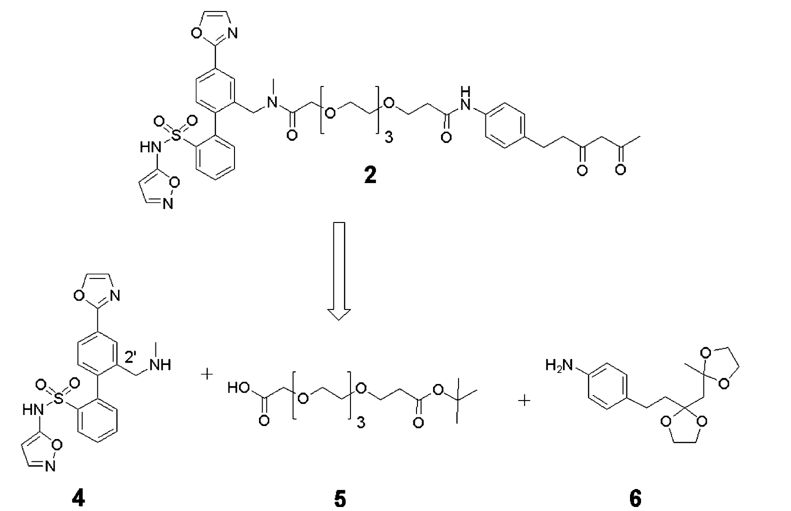


Figure 2. Design of β-diketone containing, ET_A binding pharmacophores.



Scheme 1.

convergent synthesis required three key intermediates **4**, **5**, and **6**.

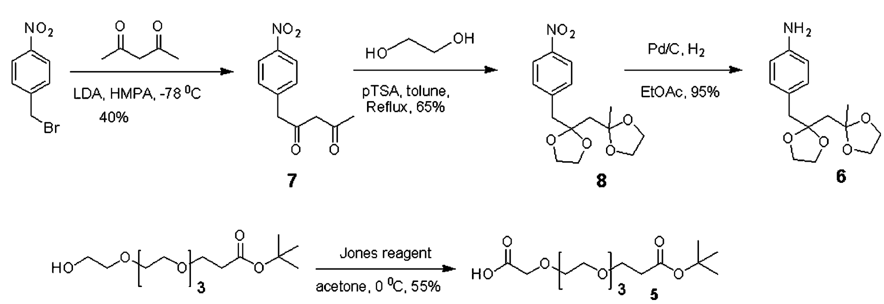
The masked diketone segment **6** was synthesized from 4-nitrobenzyl bromide as shown in Scheme 2. Treatment of 4-nitrobenzylbromide with the dianion of 2,4-pentanedione, generated with LDA at −78 °C, provided the β-diketone **7** in 40% yield.[3] The β-diketone in compound **7** was converted to bis-ketal derivative **8** before the reduction of the nitro group (Pd/C/H_2) 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)carbodiimide (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 ($A_{280}^{0.1\%} = 1.4$).

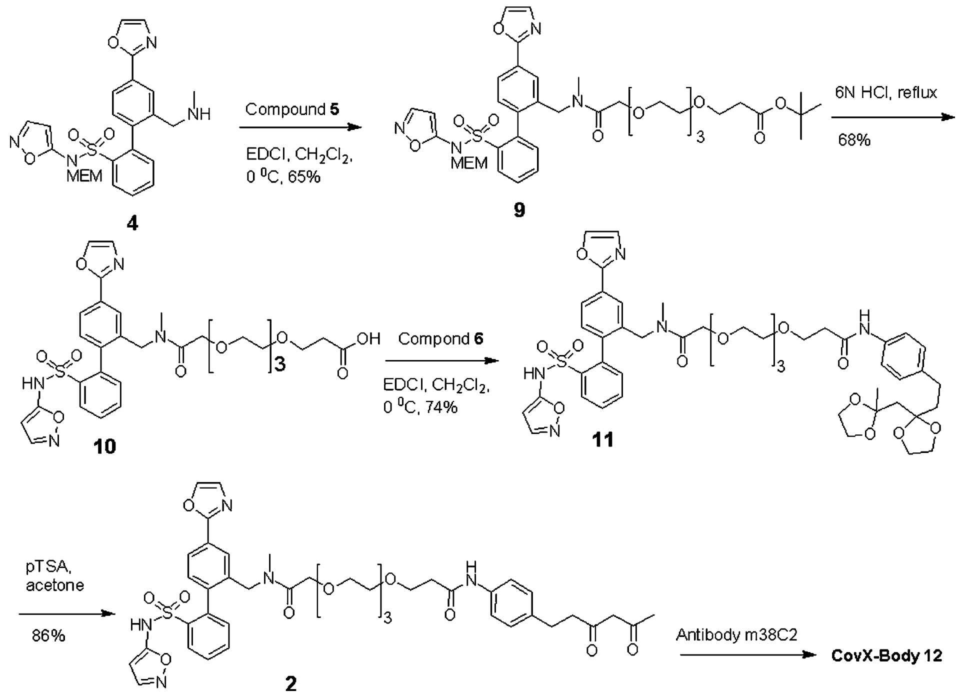
Target compound **3**, with a tether replacing the oxazole group, was synthesized as shown in Scheme 4. Mitsunobu reaction of 2-iodo, 5-hydroxy benzaldehyde with PEG alcohol provided compound **13** in 55% yield. Carbon–carbon bond formation between the boronic acid derivative[11] and compound **13** 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.

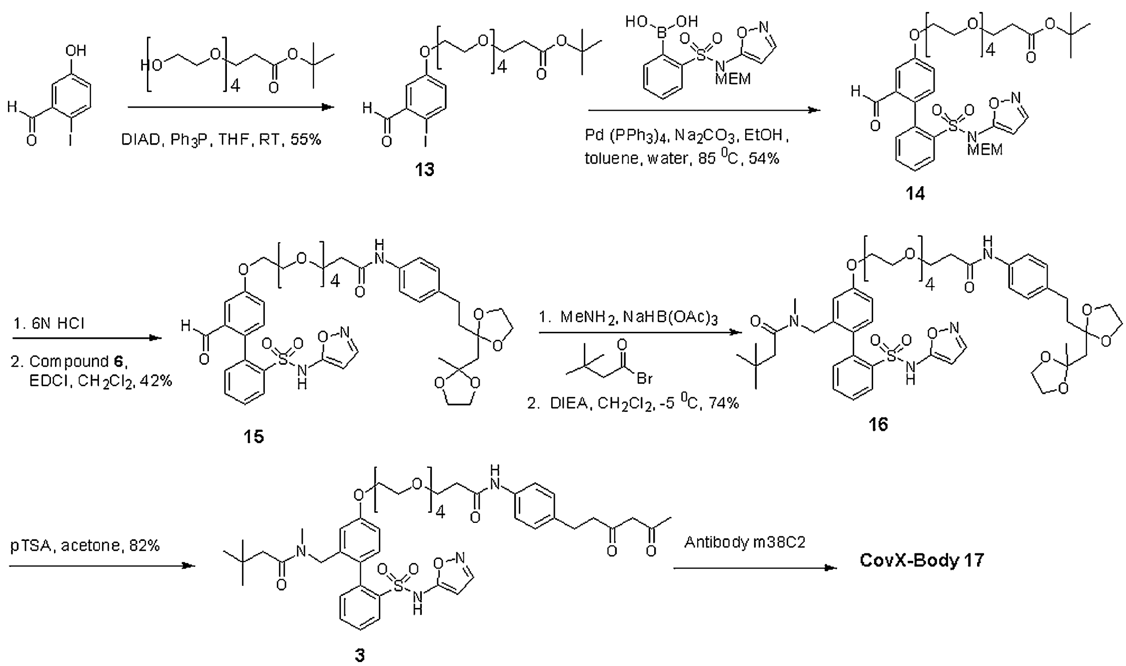
The length of the spacer, the attachment point to the pharmacophore, and the chemical compatibility be-



Scheme 2. Synthesis of protected β-diketone (**6**) and PEG diacid (**5**).



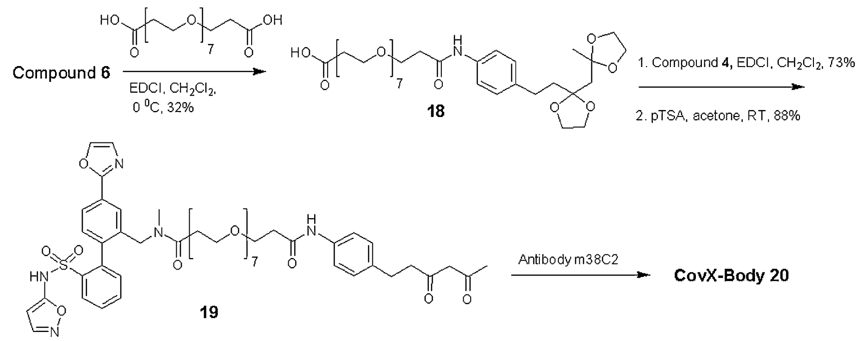
Scheme 3.



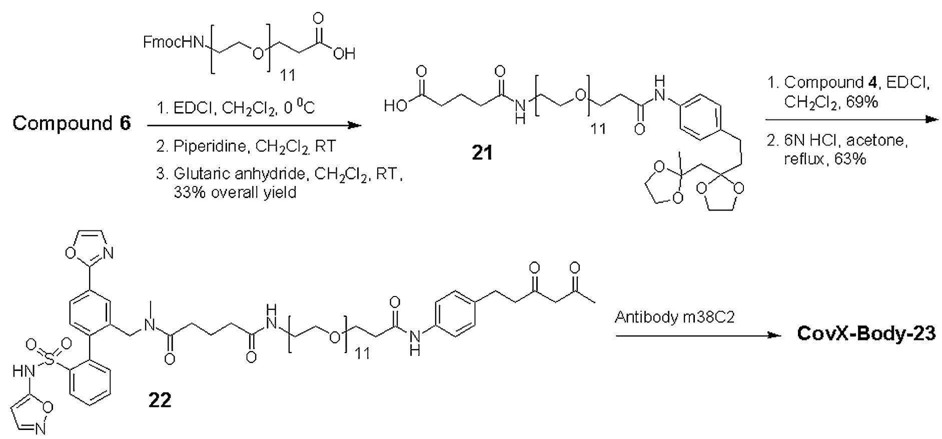
Scheme 4.

tween 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.



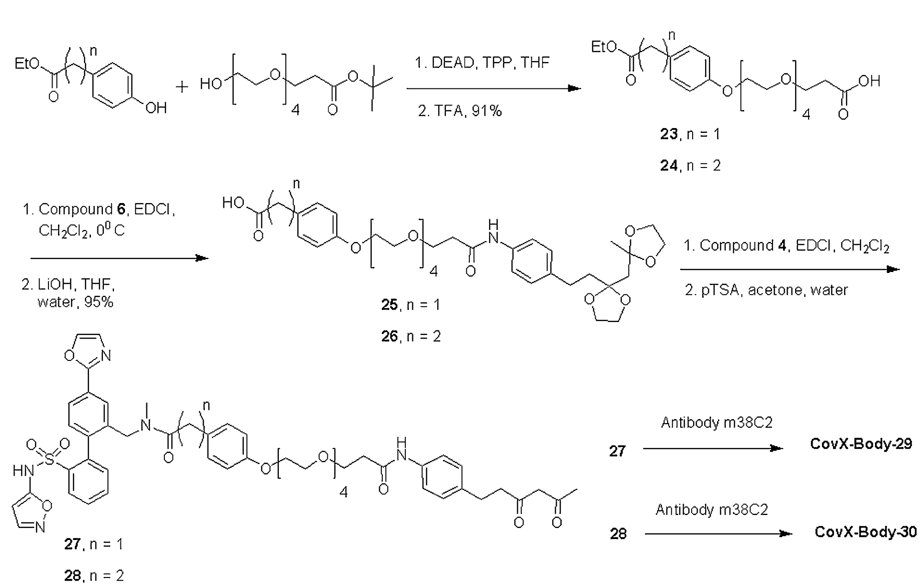
Scheme 5.



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 2′-position on ET_A/ET_B selectivity.[11] 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_A and ET_B receptor binding. The ET_A and ET_B receptor binding affinity was measured using I^{125} labeled ET-1 and CHO cells that express human ET_A



Scheme 7.

Table 1. ET_A and ET_B binding assay[a] results

Compound	ET_A IC_{50}[b] (nM)	ET_B IC_{50} (nM)	ET_B/ET_A
1	1.0	6003	8978
2	1.6	1110	1121
CovX-Body 12	8.6	4360	507
3	44.9	>10,000	
19	8.0	4690	564
CovX-Body 20	7.2	9370	1372
22	7.7	4510	564
CovX-Body 23	14	10,000	1372
27	4.6	1910	420
CovX-Body 29	6.0	570	95
28	21.6	4920	228
CovX-Body 30	15.6	5170	331

[a] The inhibition of endothelin binding to ET_A and ET_B receptors stably expressed on CHO cells was measured using ^{125}I-labeled ET-1 competitive assays.

[b] IC_{50} values were calculated using means of at least three measurements for six concentrations from 1 μM to 10 pM.

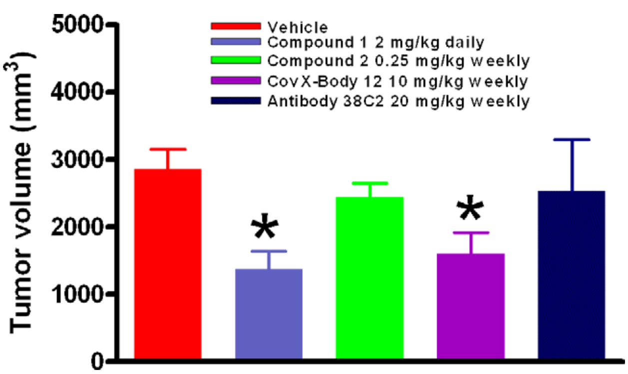


Figure 3. Anti-tumor efficacy of **CovX-Body 12** in PC-3 human xenograft. *$P < 0.05$, one-way ANOVA versus post Dunnett's multiple comparison test to vehicle.

or ET_B receptors[13,14] (Table 1). In all binding assays, the ET_A targeting CovX-Bodies, the β-diketone analog, and the parent small molecule compound **1** were evaluated. Compound **2** showed an affinity for ET_A similar to that of the parent compound **1** (IC_{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_{50} of 8.6 nM, showed a marginal loss in the ET_A binding affinity. On the other hand, the ET_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_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_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 ~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.

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