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Parallel Synthesis and Screening of Peptide Conjugates

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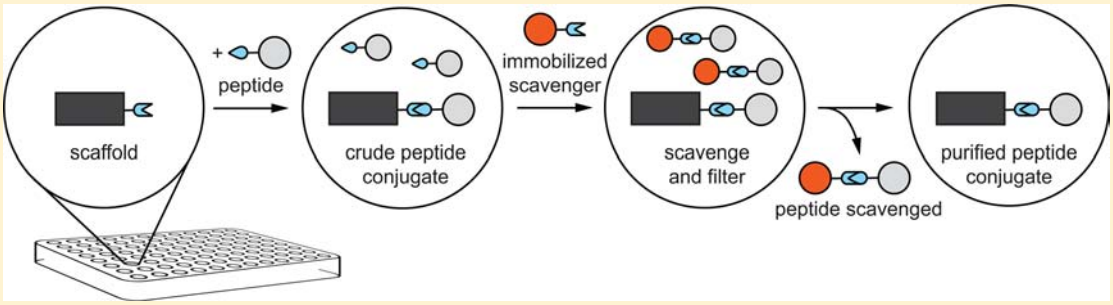
Supporting Information



ABSTRACT: Peptide conjugates represent an emerging class of therapeutics. However, in contrast to that of small molecules and peptides, the discovery and optimization of peptide conjugates is low in throughput, resource intensive, time-consuming, and based on educated decisions rather than screening. A strategy for the parallel synthesis and screening of peptide conjugates is presented that (1) reduces variability in the conjugation steps; (2) provides a new method to rapidly and quantitatively measure conversion in crude conjugation mixtures; (3) introduces a purification step using an immobilized chemical scavenger that does not rely on protein-specific binding; and (4) is supported by robust analytical methods to characterize the large number of end products. Copper-free click chemistry is used as the chemoselective ligation method for conjugation and purification. The productivity in the generation and screening of peptide conjugates is significantly improved by applying this strategy as is demonstrated by the optimization of the anti-Angiopoietin-2 (Ang2) CovX-body, CVX-060, a peptide-antibody scaffold conjugate that has advanced in clinical trials for oncology indications.

INTRODUCTION

Peptides represent a unique and emerging class of therapeutics in the pharmaceutical industry.[1,2] A key limitation in the use of peptides as therapeutic agents is their poor systemic half-life, which can often be dramatically improved by conjugation to a carrier scaffold, such as a high molecular weight polymer or a protein.[3] The carrier prevents rapid renal clearance of the peptide and also provides partial protection from proteolysis. This clinically translates in a longer duration of action and a reduction in the frequency of dosing.

Advances in both peptide chemistry and automation have enabled high-throughput, parallel synthesis of highly diverse peptide libraries.[4] Peptide libraries are routinely used to query structure activity relationships and optimize binding affinity and stability. However, conducting an optimization screen for peptide conjugates remains resource intensive and time-consuming, but is necessary as properties such as binding affinity and *in vivo* stability can significantly change upon conjugation. Commonly, the optimization and selection process for peptide conjugates is sequential and based on educated decisions rather than screening, as there are numerous hurdles to an efficient screening process. These include stability of reactants, reproducibility, and compatibility of the bioconjugation reaction across a large variety of conjugates, and the need for robust purification and analytical characterization methods for the large number of end products. As a result, the generation of a library of peptide conjugates that enables head-to-head comparisons, selection, and optimization is very challenging.[5−7]

Here, we present a strategy that addresses the significant challenges encountered in the development of a parallel synthesis approach for peptide conjugates (Scheme 1).

Key components of the strategy are the use of the following:

1. **A Robust and Chemoselective Bioconjugation Chemistry** (fully compatible with other reactive groups in biomolecules) that provides stable starting materials and end products. One of the reactive groups has to be compatible with solid phase peptide synthesis (SPPS) to allow for site-specific introduction of the linker at any position within a peptide sequence. Typically, peptide conjugates are generated with conjugation chemistries that are not chemoselective, prone to hydrolysis, and poorly compatible with SPPS. As a result, the throughput for screening peptide conjugates using these chemistries is low and significant variability in the extent of conjugation and distribution of end products is observed.

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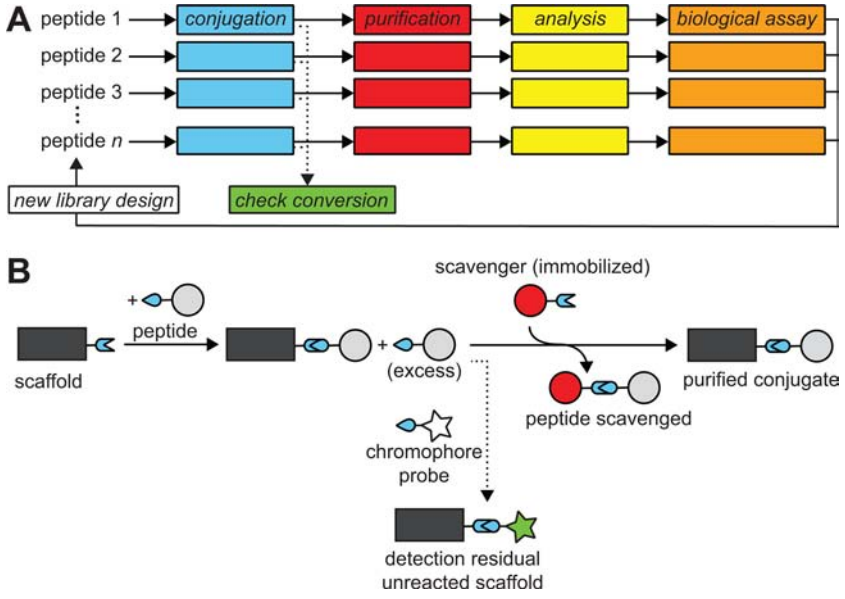
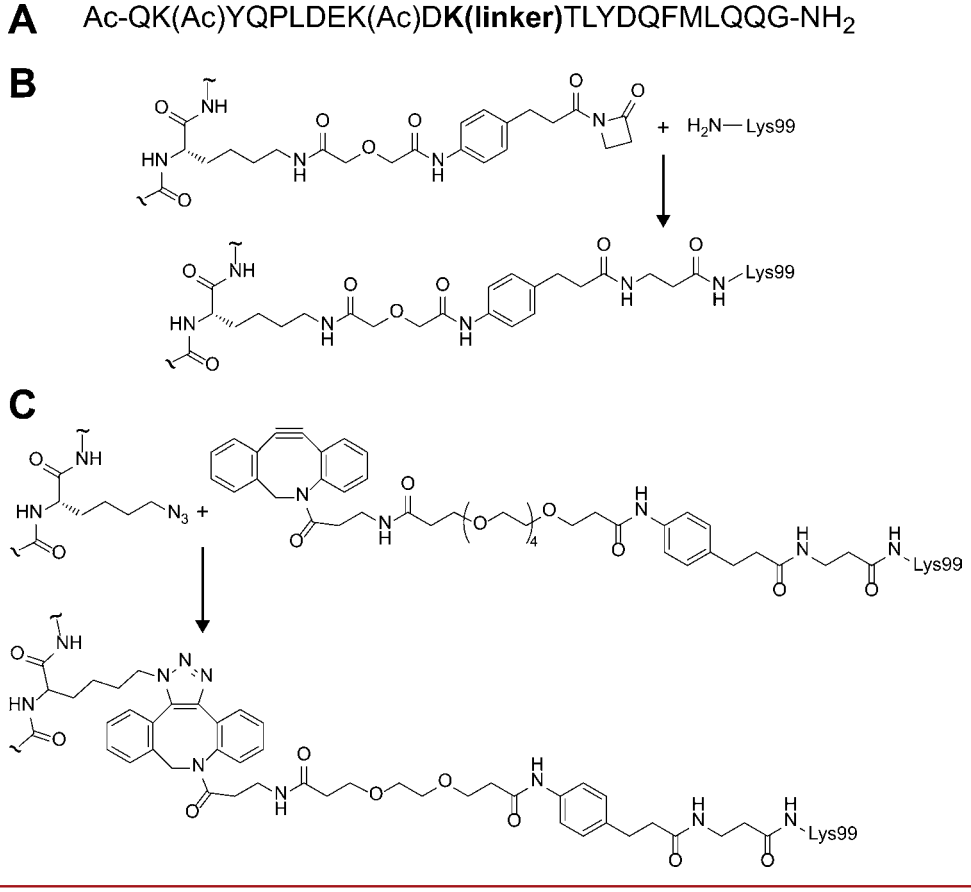
Scheme 1. (A) Steps in the Parallel Synthesis and Screening of Peptide-Conjugates; (B) General Scheme for Conjugation and Purification[a]



[a]A chromophoric assay is used to measure conversion and detect residual unreacted scaffold in a sample from crude conjugation mixture.

Scheme 2. (A) Sequence of the Ang2-Binding Peptide of CVX-060;[10] (B) Traditional β-Lactam Fusion Reaction to Lys99 in the Catalytic Pockets of the CVX-2000 antibody scaffold;[11] (C) Copper-Free Click Reaction to DBCO-Modified (at Position Lys99) CVX-2000



2. **A Single Lot of Scaffold** that is modified with the complementary reactive group for conjugation to the peptides. This eliminates variability on the scaffold as the number and position of conjugation sites are now fixed. It ensures that differences observed between the peptide

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conjugates are solely due to differences in the peptide sequences within a library.

3. **A Rapid, Quantitative Chromophoric Assay** that can be performed on samples from crude conjugation mixtures to detect residual unreacted scaffold, analogous to the ninhydrin test that is widely applied to determine conversion in coupling reactions during SPPS.[8] A chromophoric assay designed for the bioconjugation reaction of choice can provide a quick, quantitative readout of conversion. This allows for the identification of incomplete conjugation reactions early on in the process.
4. **Immobilized Scavenger for Purification** to efficiently remove excess peptide from the reaction mixture via filtration. The use of immobilized scavenging reagents that capture through chemical reaction is a well-established concept in the generation of combinatorial libraries of small molecules,[9] but has remained unexplored to increase productivity in the generation of peptide conjugates.
5. **Robust Analytical Methods** to monitor consistency and to provide the analytical characterization required before the conjugates are tested for activity.

In this manuscript we demonstrate that the productivity in the generation of peptide conjugates can be significantly improved by using the strategy described in Scheme 1 and by implementing the key components outlined above without impacting the outcome of a screen. To illustrate our approach, we chose to reproduce the peptide optimization for the anti-Angiopoietin-2 (anti-Ang2) CovX-body, CVX-060, a peptide–antibody scaffold conjugate that has advanced in clinical trials for oncology indications.[10] CovX-bodies are a novel class of biotherapeutic agents created by the site-specific conjugation of a relatively small molecular weight therapeutic to the catalytic pockets of a carrier antibody (CVX-2000) that serves as a half-life extender.[11]

RESULTS AND DISCUSSION

Optimization of the Ang2-binding peptide sequence (Scheme 2A) in the context of CVX-060 was originally done by site-specific fusion of β-lactam-equipped peptides to Lys99 in the catalytic pockets of the CVX-2000 antibody scaffold (Scheme 2B).[10,11] The limited compatibility of the β-lactam linker with nucleophilic amino acids in the peptide, such as Lys and His (Lys side chains had to be acetylated and His was excluded to prevent side reactions), the sensitivity of the linker to hydrolysis, and variability in the fusion reaction posed limitations to the throughput and extent of optimization of the Ang2-binding peptide conjugate. The conjugation reactions needed to be individually optimized and characterized. As a result, the β-lactam fusion reaction was not amenable to a parallel approach and the throughput was low.

We replaced the β-lactam fusion reaction in the peptide–antibody conjugation step (Scheme 2B) with the copper-free click reaction (Scheme 2C) (key component 1). While copper-catalyzed click chemistry has made a great impact on drug discovery, particularly on the discovery of small molecule enzyme inhibitors and through its use in activity based profiling of proteins, the reported toxicity of copper(I) has raised concerns and poses limitations on its use in the drug discovery process of bioconjugates.[12] As a result, the impact of copper-catalyzed click chemistry on bioconjugate drug discovery has been very limited.[12] Copper-free click chemistry has currently not been used to impact productivity in drug discovery, but is well positioned to do so for bioconjugate therapeutics. The copper-free click reaction between azides and constrained alkynes is chemoselective, and does not require additives or catalysts.[13,14] The reaction has been effectively used for biomolecular labeling,[15,16] for metabolic labeling of living cells,[17−22] and for labeling reactions in living animals.[23−25] Copper-free click chemistry is compatible with a wide range of buffers (pH 3–8) and cosolvents. Peptide-azides can be readily synthesized by standard SPPS protocols and *aza*-dibenzocyclooctyne (DBCO)[15,26] precursors are commercially available.

A single lot of DBCO-modified CVX-2000 antibody scaffold was synthesized at gram scale supporting the full screen. This ensures that the antibody scaffold is identical for all reactions and that the variability in the conjugation of the peptides to the antibody scaffold inherent in the β-lactam fusion reaction is resolved by replacing it with the robust copper-free click reaction (key component 2). For this, CVX-2000 was site-specifically modified with two DBCO groups by fusion of a DBCO-PEG$_5$-β-lactam linker to Lys99 in the catalytic pockets of the antibody. The final purified and fully characterized stock solution of DBCO-modified CVX-2000 was at 16.55 mg/mL (109 μM) in 10 mM His, 10 mM Gly, 2% sucrose (pH 6.5).

A library of 83 peptide-azides was designed based on the previously reported series of peptides.[10] We included the 22 originally selected sequences to allow for direct comparison of the outcome of the selection process for the two approaches. Lys at the linker position in the original peptide series was replaced by the unnatural amino acid lysine-azide (Lys(N_3)), which in contrast to the β-lactam linker was efficiently and robustly introduced during SPPS using commercially available Fmoc-Lys(N_3)-OH as a building block. The use of standard activation and coupling protocols for the implementation of the azide linker allowed for full automation of the synthesis of the peptide library. Lys(N_3) is compatible with all natural amino acids, eliminating the previous need to alter Lys and His residues.

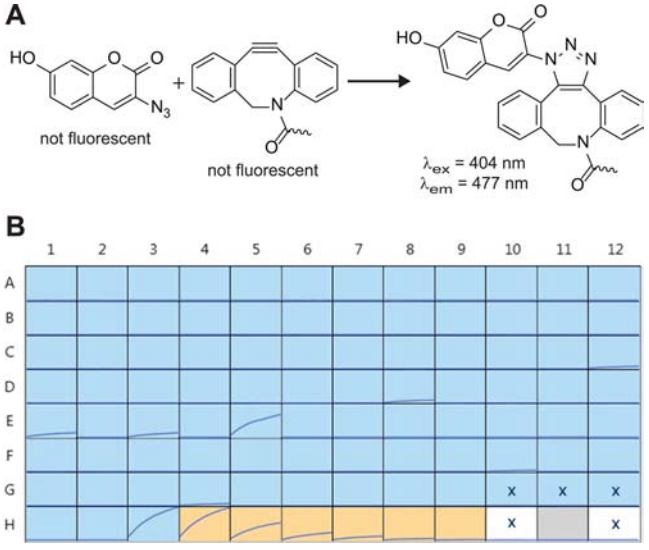
The peptide-azide library included a tether positional scan, an alanine scan to determine the amino acids important for binding, and amino acid mutations at position 9, which was identified to be important for stability. A tether positional scan was also included for the peptide with uncapped Lys instead of acetylated Lys to demonstrate the orthogonality of the method (see SI Table S2 for the complete list of sequences). The peptide-azides were generated using automated Fmoc-mediated SPPS (96-vessel block format) and purified by automated preparative reversed-phase high pressure liquid chromatography. After lyophilization, each peptide-azide was reconstituted at 3 mM in dimethyl sulfoxide (DMSO).

The synthesis of the peptide–antibody conjugates was performed in parallel on a 96-well plate and is initiated by preloading 84 wells (**A01**-**G09**, **H01**-**H03**) with 180 μL of 16.55 mg/mL stock of DBCO-modified CVX-2000 (3 mg in each well). A different peptide-azide (25 μL of 3 mM stock) was added to each well and reacted overnight at RT, with the exception of **H03** to which 25 μL of DMSO (no peptide) was added as a control. Due to the high efficiency of the copper-free click reaction at these concentrations, only a small excess of peptide-azide was required to reach full conversion.

Determining the extent of conjugation in a crude reaction mixture can be difficult, labor intensive, and sample consuming. However, it is extremely valuable to know the success rate of the individual conjugation reactions and identify outliers early

on in the process. We addressed this by developing a fluorescent assay using Coumarin-azide that rapidly and quantitatively determines conversion using only 6.7 μL samples (key component 3). Coumarin-azide, which was originally developed by Wang et al.,[27] readily reacts with alkynes to become fluorescent upon formation of the triazole linkage. The direct fluorescence readout has been of great value for the optimization of the copper-catalyzed click reaction.[28] Here we demonstrate that Coumarin-azide can be effectively used in a 96-well format to rapidly and quantitatively measure DBCO conversion in crude conjugation mixtures (Coumarin assay, Scheme 3A).

Scheme 3. (A) Reaction of Coumarin-Azide with DBCO; (B) Read-out of the Coumarin Assay Performed in Parallel for the 84 Conjugation Reactions[a]



[a] λ_{ex} = 404 nm, λ_{em} = 477 nm; every 5 min for 1 h. Wells marked X = empty, **H04-H09** (orange) = standard curve, **H11** (grey) = buffer.

In short, a 6.7 μL sample of crude conjugation mixture from each well was diluted 10-fold into 10 mM His, 10 mM Gly, 2% sucrose (pH 6.5) in a new 96-well plate. Directly after addition of 2 μL of 2.5 mM Coumarin-azide in DMSO to each well, fluorescence (λ_{ex} = 404 nm, λ_{em} = 477 nm) was measured every 5 min for 1 h in a plate-reader. An increasing signal at 477 nm indicated incomplete conversion of DBCO on the CVX-2000 antibody scaffold, while a flat line confirmed full reaction with peptide-azide. We found that the vast majority of the reactions were complete (>95% conversion) with the exception of **E05** (Scheme 3B). Further analysis of the **E05** peptide stock solution revealed that the concentration of peptide-azide was much lower than the 3 mM expected based on the weighed amount of lyophilized product. The β-lactam fusion reaction used previously is not amenable to a sensitive chromophoric assay such as the Coumarin assay, but relies on the individual analysis of each crude conjugation reaction by SEC-MS. The processing time per sample to measure conversion has been reduced from 15 min by SEC-MS in the previous study to ∼0.6 s using the Coumarin assay.

Following conjugation, residual peptide-azide was efficiently removed by using immobilized DBCO as a chemical scavenger (key component 4). For this, amine-modified agarose was reacted with DBCO-*N*-hydroxysulfosuccinimide to generate a ∼50% suspension of DBCO-modified agarose with the capacity to capture 1.2 μmol azide per mL suspension. For the purification of the peptide−antibody conjugates, 100 μL of each well was transferred to a new 96-well plate that is preloaded in wells **A01-G09** and **H01-H03** with 40 μL of the ∼50% suspension of the DBCO-modified agarose. After overnight incubation, the agarose was removed by filtration over a 96-well Zeba spin plate (Pierce). At this point the 83 peptide−antibody conjugates and the DBCO-modified CVX-2000 reference were ready for analysis and biological testing. Also, in this purification step a significant reduction in the processing time was achieved. In the previously reported method, the crude peptide conjugates required preparative SEC for purification.[10] The β-lactam chemistry is not amenable to purification with an immobilized scavenger, as excess peptide-β-lactam hydrolyzes over the course of the conjugation reaction. By being able to use a scavenger approach for the copper-free click chemistry, the purification time per conjugation reaction was reduced from ∼1 h (excluding the additional buffer exchange that is required after SEC purification) to ∼10 min (while keeping the peptide conjugates in a desirable formulation buffer during purification).

Analytical methods (key component 5) were developed to support plate-based analysis of the peptide−antibody conjugates and details on each method are provided in the Experimental Procedures. The concentration of peptide−antibody conjugate in each well was determined by measuring the absorption at 280 nm in a plate reader (SI Figure S4). Size exclusion chromatography−mass spectrometry (SEC-MS) analysis confirmed the measurements from the Coumarin assay (see for representative examples SI Figure S5). In all cases in which residual unreacted DBCO was detected in the Coumarin assay, a lower distribution of peptide on the antibody scaffold was measured by SEC-MS.

Both the amount of residual peptide-azide and the extent of aggregation of the peptide−antibody conjugates were determined by SEC. After purification the vast majority of the conjugates contained <5% residual peptide (SI Figure S6) and <6% peptide−antibody conjugate aggregates (SI Figure S7). At these levels free peptide and aggregates do not significantly affect the activity measured in the biological assay described below.

One of the selection criteria in the CVX-060 peptide optimization study was the ability of the conjugates to compete with Tie2 for binding to Ang2.[10] To allow for comparison with the original screen, the 83 copper-free clicked peptide−antibody conjugates and the unreacted DBCO-modified CVX-2000 reference were tested in the same Tie2/Ang2 competition assay that was reported previously (SI Scheme S2).[10] The copper-free clicked conjugates were ranked relative to **H02** for their ability to compete with Tie2 for binding to Ang2 (SI Table S3). **H02** has the same sequence as the Ang2-binding peptide in CVX-060, but has a PEG_4-N_3 linker instead of the β-lactam-linker at its linker position. **H02** and CVX-060 showed very similar activity in the Tie2/Ang2 competition assay. The IC_{50} of **H02** was 2.4 nM versus 1.2 nM for CVX-060 (Figure 1).

At this stage of the screen, changes are identified that can be made to the peptide sequence or linker position without being detrimental to the ability of the peptide−conjugate to compete with Tie2 for binding to Ang2. Despite the fact that the two studies were performed entirely independent using different methods for conjugation and purification, the parallel approach

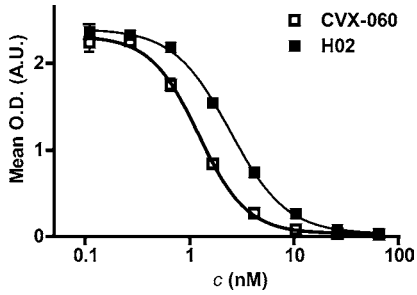


Figure 1. Full titration curves for reference standards **H02** and CVX-060 in Tie2/Ang2 competition assay.

gave a very similar outcome (Figure 2) in a much shorter time frame.[29] The peptide sequences that were similar or superior

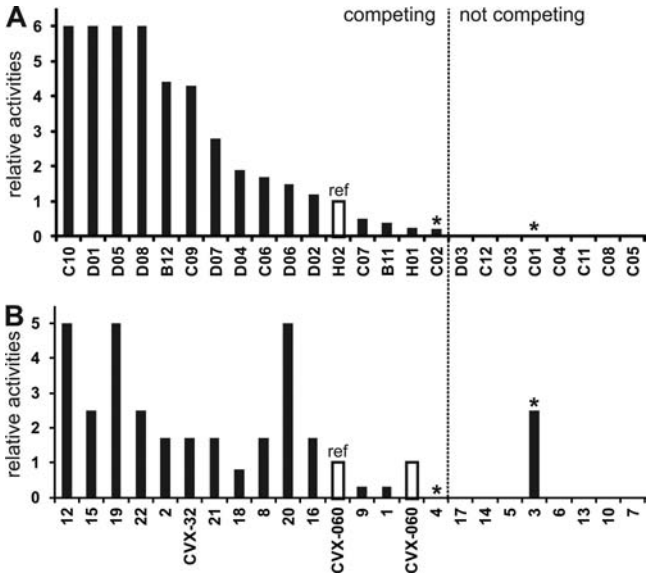


Figure 2. (A) Relative activities of the peptide conjugates in the Tie2/Ang2 competition assay.[29] The activities are normalized to the activity of reference **H02**. (B) The results of the previously reported optimization screen using the traditional approach[10] are normalized to the activity of CVX-060.[30] The peptide sequences are aligned: **C10** = **12**; **D01** = **15**; **D05** = **19**, etc., only the linker to the antibody differs. *In contrast to the original study, **C02** did show some activity in the Tie2/Ang2 competition assay and **C01** was found not to compete.

with respect to CVX-060 in the previous study (Figure 2A, competing) were found to be similar or superior with respect to **H02** in the current screen (Figure 2B, competing). Peptide sequences with mutations or changes in linker position that were detrimental in the previous study (Figure 2A, not competing) were also found to be detrimental in the current screen (Figure 2B, not competing).

Compared to the original study, only **C01** and **C02** gave a different outcome (Figure 2). **C01** was found to be inactive in the current study, while peptide **3** (equivalent sequence, but different linker chemistry) demonstrated superior competition compared to CVX-060 in the original study. To exclude sequence errors in the synthesis and major impurities, peptide **C01** was resynthesized independently 3 times, and purified carefully by RP HPLC. However, in our hands the **C01** peptide conjugate remained inactive. The closely related peptides **A03**, which has the same peptide sequence as **C01**, but without Lys acetylated, and **D11**, which has Ala at position 3 instead of the $K(N_3)$ and $K(N_3)$ at position 11, are also inactive in the Tie2/Ang2 competition assay (Table 1). **C02**, which was reported to be not competing in the original study (peptide **4**), showed activity in the current format. Also here, the closely related peptides **A04**, which has the same peptide sequence as **C02**, but without Lys acetylated, and **D12**, which has Ala at position 4 instead of the $K(N_3)$ and $K(N_3)$ at position 11, show similar activity as **C02** in the Tie2/Ang2 competition assay (Table 1).

The parallel synthesis and screening approach allows for a higher throughput and a more thorough optimization of peptide-conjugates in a shorter time frame, while minimizing variability.[31] The larger set of conjugates in the new strategy allowed for a more robust validation of the trends observed in the sequence optimization. Specifically, the tether walks show that tethering can be done at positions 2 (**A02**, **B12**), 8 (**A08**, **C06**), 11 (**A11**, **C09**), 12 (**A12**, **C10**), 15 (**B03**, **D01**), 16 (**B04**, **D02**), 18 (**B06**, **D04**), 19 (**B07**, **D05**), 20 (**B08**, **D06**), 21 (**B09**, **D07**), and 22 (**B10**, **D08**), maintaining similar or superior competition of the peptide–antibody conjugate in the Tie2/Ang2 competition assay with respect to reference **H02** (Table 1). Acetylation of Lys is not necessary to maintain activity in the Tie2/Ang2 competition assay (**A01**-**B10** compared to **B11**-**D08**; Table 1). If anything, peptide-conjugates that have free Lys trend to be slightly more competitive in most cases than their acetylated variants. The Ala scan trends with the tether walks (**D09**-**F06** compared to **A01**-**B10** and **B11**-**D08**) (Table 1). Positions that are acceptable for tethering also tolerate Ala, provided that the linker position in the Ala scan was fixed at position 11, which is also the linker position in **H02** and CVX-060. One exception is that, with a linker at position 9, activity is maintained in the Tie2/Ang2 competition assay, while an Ala mutation at the same position is not accepted. The amino acids at positions 1, 3, 4, 5, 6, 7, 10, 13, 14, and 17 do not tolerate replacement with $K(N_3)$ for tethering or with Ala, indicating that the amino acids at those positions in the original Ang2-binding peptide sequence are crucial for activity (Table 1). Mutations at position 9 are sparely tolerated (**F07**-**G09**). The original K(Ac)09L (**F07**) appears to be the only mutation that is acceptable so far. Also, K(Ac)09N (**G01**) and K(Ac)09Q (**G03**) maintain some activity. Independent synthesis, conjugation, and purification of the same peptide give reproducible results. **C09**, **E07**, and **F07** have the exact same peptide sequence. In the final Tie2/Ang2 competition assay their relative competition to **H02**, was measured to be 4.3, 4.7, and 3.4, respectively, meaning that this sequence competes ~4-fold better with Tie2 for binding to Ang2 than reference **H02**. Similarly, for **H01**, **F11**, and **F12**, their relative competition to **H02**, was measured to be 0.3, 0.2, and 0.2, respectively, meaning that they compete ~5 times worse with Tie2 for binding to Ang2 than reference **H02**.

The use of copper-free click chemistry did not affect the outcome of the peptide optimization for CVX-060. The DBCO-modified CVX-2000 control and many of the copper-free clicked peptide–antibody conjugates did not inhibit Tie2-Ang2 binding in the competition assay, demonstrating that neither the DBCO group nor the triazole linker interfered with the assay.

Interestingly, although a change in linker composition did not impact the ranking of the peptide conjugates and the outcome of the screen, it did appear to impact the absolute activity of the conjugates. Both **H01** with $K(N_3)$ and **H02** with $K(PEG_4\text{-}N_3)$ at the linker position have the same sequence as

Table 1. Head-to-Head Comparison of the Tether Walks (with the Lys Side Chain Either Free or Acetylated) and the Ala Scan[a]

	Tether walk Lys NOT Acetylated		Tether walk Lys Acetylated		Ala scan Lys Acetylated	
AA position	Well ID	Relative competition (normalized to H02)	Well ID	Relative competition (normalized to H02)	Well ID	Relative competition (normalized to H02)
1	A01	0.6	B11	0.4	D09	0.4
2	A02	> 6	B12	4.4	D10	2.2
3	A03	0.05	C01	0.05	D11	0.05
4	A04	0.3	C02	0.2	D12	0.2
5	A05	0.05	C03	0.05	E01	0.2
6	A06	0.05	C04	0.05	E02	0.05
7	A07	0.05	C05	0.05	E03	0.05
8	A08	2.6	C06	1.7	E04	1.0
9	A09	0.7	C07	0.5	E05	0.05
10	A10	0.05	C08	0.05	E06	0.05
11	A11	0.7	C09	4.3	E07	4.7
12	A12	> 6	C10	> 6	E08	> 6
13	B01	0.05	C11	0.05	E09	0.05
14	B02	0.05	C12	0.05	E10	0.05
15	B03	4.0	D01	> 6	E11	0.2
16	B04	> 6	D02	1.2	E12	1.3
17	B05	0.05	D03	0.05	F01	0.1
18	B06	0.9	D04	1.9	F02	0.1
19	B07	> 6	D05	6.0	F03	3.5
20	B08	5.1	D06	1.5	F04	2.3
21	B09	6.0	D07	2.8	F05	0.9
22	B10	> 6	D08	6.0	F06	2.4

[a]The amino acid (AA) position number indicates at what position in the Ang2 binding peptide the K(N_3) linker or Ala is introduced. The linker position in the Ala scan is at AA position 11. In green are the positions that were identified to be acceptable for tethering.

CVX-060. A 4-fold loss in activity is observed for **H01**, while **H02** and CVX-060 have a similar IC_{50}. This implicates that once the lead sequences are determined, linker optimization would be the next step to further optimize the conjugates, not only for activity, but also for further drug development.

By being able to test a larger number of peptide conjugates in parallel we were able to identify an additional 17 sequences that competed equally or better with Tie2 for binding to Ang2 than reference **H02** (see SI Table S3). The larger set of conjugates that could be assessed using the new approach allowed for a more robust validation of the trends observed in the sequence optimization. At this point competing peptide conjugates could be taken forward for further validation, for linker optimization, or, together with the outliers from this screen, funnel back into the design of the next library of peptides to drive the sequence optimization.

CONCLUSIONS

The strategy presented here is the first demonstration of a robust parallel synthesis and screening approach for peptide-conjugates to support rapid optimization of this class of biotherapeutics in drug discovery. We envision that the strategy will be widely applicable and can be expanded to other classes of bioconjugates. It is designed not to rely on purification methods typically used for protein and antibody conjugates to make it amenable to other classes of scaffolds and biomolecules. Although we selected the copper-free click reaction in this example, other chemoselective bioconjugation methods should also be well suited to support such a strategy and significantly increase the productivity in the synthesis and optimization of bioconjugates of therapeutic interest.

EXPERIMENTAL PROCEDURES

Solvents and Starting Materials. Unless stated otherwise, all reagents and solvents were purchased from commercial sources and used without further purification. Amino acid derivatives were obtained from Aapptec (Louisville, KY, USA) and the solvents for peptide synthesis and purification from Fisher Scientific (Pittsburgh, PA, USA). Coumarin-azide was generously provided by S. Presolski and Professor M. G. Finn (The Scripps Research Institute, La Jolla, CA; current: Georgia Tech, Atlanta, GA).

Instrumentation. Peptides were synthesized on a SYRO multiple peptide synthesizer (MultiSynTech GmbH, Witten, Germany). Reversed phase high pressure liquid chromatography (RP HPLC) was performed on a Waters HPLC, equipped with a 3100 Mass Detector and MassLynx 4.1 software, using a Luna C18, 5 μ, 50 × 4.6 mm column (Phenomenex, Torrance, USA) for analytical runs and a Luna C18, 5 μ, 100 × 21.2 mm column (Phenomenex, Torrance, USA) for purification. Size exclusion chromatography (SEC) (prep scale) was performed on an AKTA Avant (GE Healthcare).

Peptide Synthesis. Peptides were synthesized on a SYRO multiple peptide synthesizer (MultiSynTech GmbH, Witten, Germany) employing standard Fmoc/*t*Bu chemistry. Amino acid derivatives were activated by 2-(6-chloro-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU). The protocol used to synthesize the peptides in the 96-vessel block format is provided in SI Table S1. In short, 5−10 mg of TentaGel S-RAM resin (loading: 0.26 mmol/g; Rapp Polymere, Tubingen, Germany) were loaded in clear Top-Line Filter Tips 1−30 μL (Micropore Filter Technology). Activation was performed *in situ* by mixing 80 μmol amino acid derivative, 80 μmol HCTU, and 40 μmol *N*-methylmorpholine

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(NMM) into each reaction vessel. Each amino acid derivative was coupled twice for 20 min. Removal of the N-terminal Fmoc-group was achieved with a solution of 20 vol % piperidine in dimethylformamide (DMF). At the end of the synthesis the peptide-resins were washed three times with 200 μL of dichloromethane (DCM) and dried under vacuum. Peptides were cleaved with a solution of 5 vol % water, 5 vol % phenol, and 2.5 vol % triisopropylsilane in trifluoroacetic acid (TFA) for 2 h and precipitated with chilled diethyl ether.

Peptide Library Design. The sequence of the Ang2-binding peptide of CVX-060 is **Ac-QK(Ac)YQPLDEK(Ac)-DK(linker)TLYDQFMLQQG-NH_2** with a β-lactam linker at the linker position.[10]

In the original publication K(Ac) at position 9 was mutated with L in the optimization series.[10] Here, we have applied the same K(Ac)09L mutation in the optimization series in order to allow for direct comparison of the outcomes of the screens. The amino acid sequences of the 83 peptide-azides that were synthesized and their recovered purified yields are listed in SI Table S2.

The peptide sequences include: (1) Tether walk, Lys not acetylated (free amine side chain); (2) Tether walk, Lys acetylated; (3) Ala scan, Lys acetylated; (4) Mutants at position 9, Lys acetylated; (5) Reference peptide-azides (that have the same peptide sequence as CVX-060, but with an azide linker instead of a β-lactam at the linker position).

Peptide Purification. The dried peptides were suspended in 500 μL of dimethyl sulfoxide (DMSO) and analyzed on a Waters HPLC equipped with a 3100 Mass Detector and MassLynx 4.1 software. Analytical runs were performed on a Luna C18, 5 μ, 50 × 4.6 mm column (Phenomenex, Torrance, USA) using a gradient 5−95% aqueous acetonitrile (ACN) (0.1% TFA) for 5 min. Purification was achieved by loading the individual peptides on a Luna C18, 5 μ, 100 × 21.2 mm column (Phenomenex, Torrance, USA) using a gradient of 35−45% aqueous ACN (0.1% TFA) for 10 min. The collected fractions were lyophilized, weighed, and stored at +4 °C. The recovered purified yield of each peptide is listed in SI Table S2.

Synthesis of the *aza*-Dibenzocyclooctyne Linker, DBCO-PEG_5-β-lactam (SI Scheme S1). Pentafluorophenol ester-PEG_5-β-lactam (PFP-PEG_5-β-lactam) (0.11 g, 0.15 mmol, BroadPharm, San Diego, CA) was dissolved in ACN (1 mL) and reacted with 1.1 equiv (0.048 g, 0.17 mmol) of *aza*-dibenzocyclooctyne-amine (DBCO-amine) (Click Chemistry Tools, Scottsdale, AZ) for 30 min at RT. The reaction mixture was purified by preparative RP HPLC over a C18 column (gradient: 30−40% aqueous ACN in 22 min, without TFA in the mobile phase to prevent amide hydrolysis at the DBCO ring that gives a COOH-PEG_5-β-lactam byproduct; flow 24 mL/min) to obtain DBCO-PEG_5-β-lactam in 48% yield. The purity of the DBCO-PEG_5-β-lactam linker was 92% and free of the undesired COOH-PEG_5-β-lactam byproduct as determined by analytical RP HPLC (Agilent Zorbax 300SB-C8 column 5 μm (2.1 × 150 mm); 2 min at 3% followed by a gradient: 3−95% aqueous ACN (0.1% TFA) in 18 min) (Supplementary Figure S1). Calcd molecular weight $[M + H]^+$: 797.9; found 797.4.

Synthesis of the DBCO-Modified CVX-2000 Antibody Scaffold (CVX-2000-PEG_5-DBCO). 5.263 mL of a 6.6 mM stock solution of DBCO-PEG_5-β-lactam in DMSO was added to 100 mL of a 20 mg/mL solution of CVX-2000 (produced in-house at CovX Research, Pfizer Inc.) in 10 mM His, 10 mM Gly, 2% sucrose (pH 6.5), gently mixed, and reacted overnight at RT. CVX-2000-PEG_5-DBCO was purified by SEC over a 2.9 L Superdex 200 column equilibrated with SEC elution buffer (0.15 M sodium phosphate, 0.5 M sodium chloride (pH 7.0)). Elution fractions containing monomeric CVX-2000-PEG_5-DBCO were dialyzed separately into 10 mM His, 10 mM Gly, 2% sucrose (pH 6.5) using up to six 50 kDa 15 mL Amicon centrifugal units per sample fraction. All samples were vacuum filtered (0.2 μm filter) and pooled after analysis by SEC-MS. The final concentration of the pooled fractions was 25.54 mg/mL (determined by UV at 280 nm; absorption of a 1 mg/mL solution of CVX-2000-PEG_5-DBCO at 280 nm is 1.47). The scaffold was diluted with 10 mM His, 10 mM Gly, 2% sucrose (pH 6.5) to 16.55 mg/mL and stored at +4 °C. Recovered yield was 1.2 g (60%). Calcd molecular weight: 15 1190; Found: 15 1160. See SI Figure S2 for the SEC-MS spectrum of CVX-2000-PEG_5-DBCO.

Synthesis of Reference Peptide H02. A solution of Ang2-binding peptide Ac-QK(Ac)YQPLDEK(Ac)DK(NH_2)-TLYDQFMLQQG-NH_2 (51 mg, 1 equiv) in DMSO (0.75 mL), containing *N*,*N*-diisopropylethylamine (DIEA) (13 μL, 4 equiv), was added dropwise to a solution of azide-PEG_4-NHS ester (Click Chemistry Tools, Scottsdale, AZ, 11 mg, 2 equiv) in DMSO (0.75 mL), while stirring at RT. After 30 min the **H02** reference peptide was purified by RP HPLC over a C18 column (gradient: 25−35% aqueous ACN (0.1% TFA) in 22 min; flow 25.6 mL/min) and obtained in 35.7 mg (63.6%) yield. Calcd molecular weight: 3116.4; Found: 3116.4.

Synthesis of DBCO-Modified Agarose (Immobilized Scavenger). 2 mL of CarboxyLink Coupling Gel (Thermo Scientific; 4% cross-linked beaded agarose supplied as a 50% slurry containing 0.02% sodium azide; activation level of 16−20 μmol amine/mL of gel) was centrifuged and the storage solution was decanted. The agarose was thoroughly washed with PBS (10 × 5 mL) to remove sodium azide. The approximately 50% slurry of CarboxyLink Coupling Gel in PBS was subsequently reacted overnight at RT with DBCO-*N*-hydroxysulfosuccinimide (DBCO-*sulfo*-NHS) (Click Chemistry Tools, Scottsdale, AZ) (25 mg in 400 μL DMSO). The DBCO-modified agarose was thoroughly washed with 30 vol % DMSO in PBS (4 × 13 mL) and subsequently with 10 mM His, 10 mM Gly, 2% sucrose (pH 6.5) (4 × 13 mL). After the last wash, excess buffer was removed to give an approximately 50% slurry of DBCO-modified agarose in 10 mM His, 10 mM Gly, 2% sucrose (pH 6.5).

The scavenging capacity of the DBCO-modified agarose was determined by incubating 100 μL of a 0.3 mM stock solution of reference peptide **H02** (prepared through 10-fold dilution of 3 mM **H02** in DMSO into 10 mM His, 10 mM Gly, 2% sucrose (pH 6.5)), overnight at RT with either 50 μL, 20 μL, or 10 μL of the approximately 50% slurry of DBCO-modified agarose. The DBCO-modified agarose was spun down and residual **H02** in supernatant was measured by RP HPLC (Agilent 1100) using a Zorbax 300SB-C8 2.1 × 150 mm column (Agilent) (gradient: 3−95% aqueous ACN (0.1% TFA) in 20 min; flow: 0.4 to 0.6 mL/min; data collection at 214 nm) and by SEC (Agilent 1100), using a TSK Super SW3000 4.6 × 300 mm column (Tosoh) with 200 mM potassium phosphate, 250 mM potassium chloride, 15% 2-proponal, pH 6.95 at 0.2 mL/min, and data collection at 214 nm. The scavenging capacity of the DBCO-modified agarose resin was determined to be 1.2 μmol of peptide-azide per mL suspension.

Parallel Synthesis of the Antibody−Peptide Conjugates Using Copper-Free Click Chemistry. A 96-well plate

was loaded with 180 μL of CVX-2000-PEG$_5$-DBCO (16.55 mg/mL in 10 mM His, 10 mM Gly, 2% sucrose (pH 6.5)) in wells **A01-G09** and **H01-H03**. **G10-G12** and **H04-H12** were left empty for controls in the analytical and biological assays. A stock solution of 8.1 mg/mL in DMSO (approximately 3 mM; molecular weight of the peptides is around 2800 Da) was prepared for each peptide. Then, 25 μL of peptide stock solution was added to each well: peptide **A01** to well **A01** containing CVX-2000-PEG$_5$-DBCO scaffold, peptide **A02** to **A02**, peptide **A03** to **A03**, etc. After each addition, the reaction mixture was gently mixed using a pipettor. The reactions were left at RT overnight.

Coumarin Assay. The assay is based on the formation of a fluorescent triazole-linked conjugate upon reaction of the nonfluorescent dye Coumarin-azide with the DBCO alkyne. For the assay, a 6.7 μL sample of crude conjugation mixture from each well is diluted 10-fold into 10 mM His, 10 mM Gly, 2% sucrose (pH 6.5) in a new 96-well plate. Directly after addition of 2 μL of 2.5 mM Coumarin-azide in DMSO to each well, fluorescence (λ_{ex} = 404 nm, λ_{em} = 477 nm) is measured every 5 min for 1 h in a Spectra Max M5e plate reader. A standard curve from a 2-fold serial dilution of CVX-2000-PEG$_5$-DBCO in 10 mM His, 10 mM Gly, 2% sucrose (pH 6.5) (SI Figure S3) was used to calculate the percentage of residual reactive scaffold in the crude conjugation mixtures.

Parallel Purification of the Copper-Free Clicked CVX-2000-PEG$_5$-DBCO-peptide Conjugates. A 96-well plate is preloaded in wells **A01-G09** and **H01-H03** with 40 μL of the 50% slurry of DBCO-modified agarose in 10 mM His, 10 mM Gly, and 2% sucrose (pH 6.5) (scavenging capacity of 1.2 μmol peptide-azide per mL suspension) using a single channel pipettor (part of the pipet tip was cut off to facilitate pipetting of the DBCO-modified agarose slurry). During the preloading of the 96-well plate, the DBCO-modified agarose slurry is continuously mixed to keep the suspension as homogeneous as possible and to ensure an equal distribution of beads across wells. Subsequently, 100 μL of reaction mixture is added to each well using a 12-channel pipettor: conjugation mixture from well **A01** to well **A01** containing beads, **A02** to **A02**, **A03** to **A03**, etc. The 96-well plate was gently mixed on a vortex at RT overnight. The beads settled overnight, and to ensure complete removal of the DBCO-modified agarose beads, 100 μL of each reaction mixture was filtered over a 96-well Zeba spin plate (Pierce, 7 kDa MWCO; processed according to the manufacturer's instructions) and collected into a new 96-well plate.

Intact Mass Measurements for the Peptide−Antibody Conjugates by LC-MS. Samples (5 μg injection load) were injected onto a polymeric PLRP-S column (250 × 1 mm, 5 μm, 1000 Å, Higgins Analytical) with an Agilent 1290 UPLC, using 50% aqueous ACN (0.1% formic acid) as mobile phase at 0.1 mL/min. The method was isocratic for 5 min, with the peptide−antibody conjugate eluting within the first 2 min and being directed to the Agilent 6550 Q-TOF. Electrospray conditions consisted of a gas temperature at 325 °C, capillary voltage at 4000 V, and fragmentor at 400 V. The m/z mass range was 500−4000 Da. Acquisition software was MassHunter (Agilent) and deconvolution software was BioConfirm (Agilent). Deconvolution parameters consisted of a signal-to-noise threshold of 10, mass step 1 Da, and a peak width of 1 m/z for 25 iterations.

Determination of the Final Peptide−Antibody Conjugate Concentration. A 5 μL sample from each well was diluted 20-fold with 10 mM His, 10 mM Gly, 2% sucrose (pH 6.5) in a new 96-well plate. Absorbance at 280 nm was measured using a Spectra Max M5e plate reader (Molecular Devices) within 15 min. A serial dilution of CVX-2000-PEG$_5$-DBCO was used as a standard curve. The concentration of 5 randomly selected samples was confirmed by NanoDrop analysis (Thermo Scientific).

Aggregate and Residual Peptide-Azide Analysis. Samples were analyzed for high molecular weight species/aggregates (HMW) and residual peptide-azide levels by SEC, using a TSK Super SW3000 4.6 × 300 mm column (Tosoh) with 200 mM potassium phosphate, 250 mM potassium chloride, 15% 2-proponal, (pH 6.95) at 0.2 mL/min (30 min); data collection at 280 nm for HMW and 214 nm for residual peptide-azide. Residual peptide levels were calculated based on peak area as compared to a standard curve generated from a 2-fold serial dilution of peptide **H02**.

Tie2/Ang2 Competition Assay. The competition assay was performed as previously described.[10] To enable rapid throughput and screening, single dilutions of the antibody−peptide conjugates were tested in the Tie2/Ang2 competition assay instead of a full dilution curve. The data are normalized with respect to **H02**. Briefly, antibody−peptide conjugates were diluted to 1:10 000 using two 0.5 mL deep 96-well dilution plates, and tested in the Tie2/Ang2 competition assay. CVX-060 was used as a reference standard, and the antibody−peptide conjugate concentrations were extrapolated from the standard curve. Concentrations of each antibody−peptide conjugate were then normalized for their respective protein concentrations, by dividing by the previously determined absorption at 280 nm.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Protocol for peptide synthesis in the 96-vessel block format, list of peptide sequences and their purified recovered yields, and additional figures, schemes, and tables described in the text. This material is available free of charge via the Internet at http://pubs.acs.org/.

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Notes

The authors declare the following competing financial interest(s): The authors are (A.D.) or were (M.M., G.D.I., M.J.M., M.B., N.S., S.C., A.B.) employees of Pfizer Inc. for this work.

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(29) The absolute values (*x*-axis of Figure 2A and B) should not be compared. The Tie2/Ang2 competition assay was not done head-to-head, but years apart. The two studies were performed entirely independent using different methods for conjugation and purification. The Tie2/Ang2 competition assay has its intrinsic variability. It was also simplified for the current study to increase throughput (single dilutions were measured instead of full dilution curves). However, the format of the assay is identical in both studies (SI Scheme S2) and therefore the general trends observed in both studies should hold.

(30) In the original study the IC_{50} (nM) of each peptide-antibody is reported.[10] We normalized the IC_{50} values to the IC_{50} of CVX-060 (0.5 nM) to allow for comparison (SI Table S4).

(31) A flowchart indicating which steps presented in this manuscript can be done in parallel and the timelines associated with those steps is provided in SI Scheme S3.