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COMMUNICATION

Late-Stage Peptide Modification with Salicylaldehyde Tag Enhances Affinity for Nuclear Factor-kappa B Essential Modulator

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The late-stage peptide functionalization with salicylaldehyde (SA) tags is described here as a versatile design of potential Lys-engaging, reversible-covalent ligands. This approach was applied to a known binder for NEMO, a protein involved in the activation of the pro-inflammatory transcription factor NFκB. Fluorescence anisotropy screening led to the identification of SA-tagged peptides with higher affinity than the wild-type sequence.

Introduction

In recent years, growing attention has been devoted to the identification of peptide and peptidomimetic ligands capable of selectively targeting proteins of interest,[1] particularly those forming covalent bonds with their targets.[2] This emerging class of covalent peptide binders offers unique opportunities for designing potent and durable modulators of protein function. Salicylaldehyde (SA) derivatives have emerged as powerful amine-engaging units and, stimulated by the high frequency of Lys residues in the proteome,[3] they have been incorporated in various small molecule drugs,[4] glycomimetic[5] and peptide[6] ligands.

In the latter context, considering that protein-peptide binding interactions typically span a wide interface area, the precise insertion of the SA tag at suitable peptide positions could be crucial to covalently engage a specific Lys(ε-NH_2) group of the target protein. Ideally, this additional covalent interaction could synergize with the original non-covalent forces, resulting in a highly stable ligand-protein complex.

With this aim, we recently reported on the synthesis of non-coded amino acids, substituted the homoserine side chain with a SA tag. These new scaffolds, endowed with suitable protecting groups, could be inserted in primary sequences during peptide synthesis.[7] Unfortunately, these modified amino acids could be obtained only with long and inefficient multistep synthetic sequences, which strongly limit their use as building blocks for peptide production. For this reason, we looked at more practical and versatile design of SA-tagged peptides.

"Click" chemistry approaches, such as the copper-catalysed azide-alkyne cycloaddition (CuAAC), offer unique opportunities to connect two highly functionalized molecules in an efficient and chemoselective way.[8] In particular, CuAAC proved a robust approach to connect the azido group in biomolecules (*i.e.* nucleic acids[9] and peptides *N* and *C-termini*[6]) to alkyne-bearing SA derivatives, devoid of protecting groups on the aldehyde and phenol units.

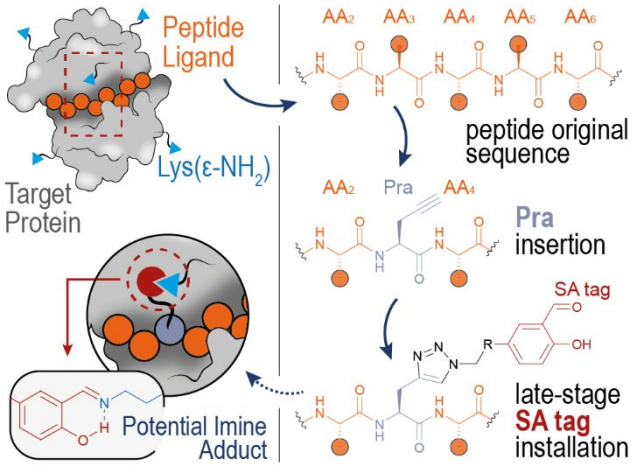

Figure 1. Design of potential reversible-covalent peptide binders through tailored insertion of a propargylglycine residue (Pra) at suitable positions of the peptide original sequence, followed by installation of the salicylaldehyde (SA) tag through CuAAC. Ideally, the resulting SA-tagged peptide form an imine adduct with the amino group of a specific Lys residue close to the peptide binding site, stabilizing the peptide-protein complex.

Propargylglycine (Pra) is a non-coded amino acid featuring a terminal alkyne group on its side chain: its *N*-Fmoc protected version is commercially available in both enantiomeric forms, enabling the insertion of alkyne units in peptide sequences through solid phase peptide synthesis (SPPS).

Inspired by the high versatility of the Pra amino acid, we designed a practical strategy for the preparation of potential

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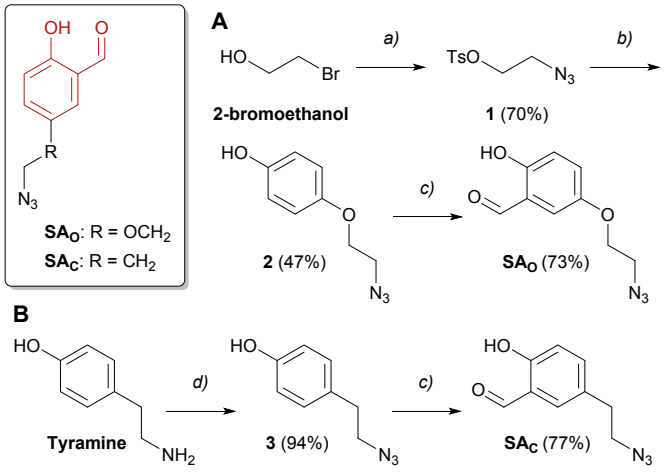
Lys-engaging peptides (Figure 1), consisting in the Pra insertion within the peptide sequence and subsequent installation of the SA tag through CuAAC.

A known peptide-protein interaction was selected as a case study to verify the feasibility of our approach, demonstrating that the generation of a panel of SA-tagged peptides and their screening against the protein of interest can lead to hit compounds with enhanced binding affinity compared to the wild-type peptide sequence.

Results and Discussion

Two SA derivatives bearing an azido group were designed and prepared as highlighted in Scheme 1. In particular, the azido group was connected to the aromatic ring through either a phenol ether bond (**SA$_O$**) or a short alkyl chain (**SA$_C$**). **SA$_O$** was prepared starting from 2-bromoethanol, which was transformed into the corresponding azide through an S_N2 reaction and then treated with tosyl chloride, to give compound **1** in good yields. Following the same strategy reported by our group in 2021,[10] the tosyl ester was reacted with hydroquinone, to give ether **2**, and then subjected to a regioselective phenol *ortho*-formylation under the Skattebøl conditions. The second SA-azide unit (**SA$_C$**) was prepared in only two synthetic steps, starting from commercially available tyramine. Following a published protocol,[11] the diazo-transfer reagent imidazole-1-sulfonylazide was prepared as a hydrogen sulphate salt, which can be handled safely as a solid. This reagent converted tyramine into the corresponding azide **3** in excellent yield (94%). Finally, a phenol *ortho*-selective formylation led to the desired SA-azide derivative **SA$_C$**. The detailed reaction procedures and analytical data are included in the Supplementary Information.



Scheme 1. Synthesis of SA-azide derivatives bearing either a phenol ether (**SA$_O$**) or a methylene (**SA$_C$**) spacer. REAGENTS AND CONDITIONS: *a)* [1] NaN_3, THF, H_2O, 0 °C to 50 °C, overnight; [2] TsCl, Et_3N, CH_2Cl_2, 0 °C to r.t., overnight; *b)* Hydroquinone, K_2CO_3, Bu_4NI, DMF, 80 °C, overnight; *c)* Paraformaldehyde, anhydrous $MgCl_2$, dry Et_3N, dry THF, 70 °C, 4 h; *d)* Imidazole-1-sulfonylazide·HSO_4^-, $NaHCO_3$, $CuSO_4{\cdot}5H_2O$, MeOH, r.t., overnight.

To compare the reactivity of aldehydes **SA$_O$** and **SA$_C$** towards imine bond formation in aqueous solution, a nuclear magnetic resonance (NMR) experiment was performed, following published protocols.[9,12] Stock solutions of the SA-azide derivatives in DMSO-d_6 were diluted in phosphate buffer prepared in deuterium oxide (pH 7.4), and ^{1}H-NMR spectra were recorded both in the presence or absence of N_α-acetyl-lysine in 10-fold molar excess. As shown in Figure S1 (Supplementary Information), after 1 h incubation at room temperature, the presence of Lys in the mixture led to approx. 33% conversion of both aldehydes into the corresponding imines, indicating that the **SA$_O$** and **SA$_C$** are similarly reactive toward the Lys(ε-NH_2) group.

The synthesized SA-azide derivatives were then installed in a model peptide binder. As a case study, we selected the protein-protein interaction (PPI) between the Ser/Thr-specific IκB kinase (IKK) and the Nuclear Factor-kappa B Essential Modulator (NEMO), which represents an important step in the activation of the pro-inflammatory transcription factor NFκB. Given this relevant role in the inflammatory signalling cascade, the IKK:NEMO PPI has been identified as an attractive clinical target and as subject of various peptide discovery campaigns.[13] The TALDWSWLQTE sequence of IKKβ proved a fundamental player of this PPI, leading to the identification of this 11-mer fragment as NEMO-binding peptide (NBP).[14] Interestingly, while NBP does not include Lys residues, the crystal structure of the NEMO-IKKβ complex revealed the presence of three Lys(ε-NH_2) groups in NEMO, proximal to the interface with the 11-mer IKKβ fragment (Figure 2).[15]

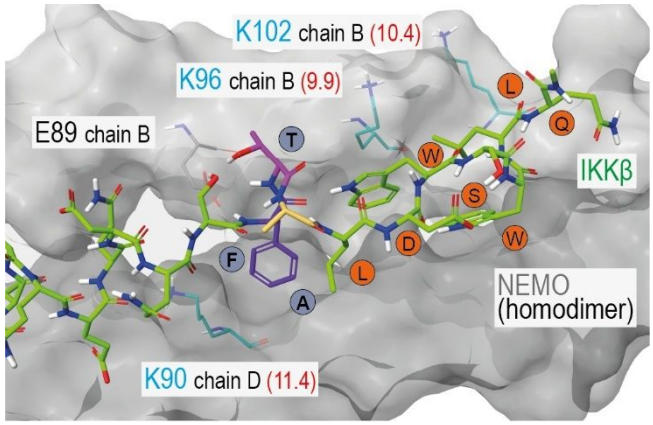


Figure 2. Crystal structure of the NEMO-IKKβ complex (PDB: 3BRV). IKKβ residues 727-743 (NEMO binding domain, NBD, chain A) are shown in green stick representation, with different colours for F734 (purple), T735 (magenta), A736 (yellow). The single chains of homodimeric NEMO are shown with surface representation, while NEMO E89 and Lys residues close to the interface with IKKβ NBD (K90 in NEMO chain D, K96 and 102 in chain B) are shown in stick representation. The figure was obtained from the published PDB and originally-edited with Schrödinger Maestro graphical interface (Schrödinger Release 2025-3). pK_a values of Lys(ε-NH_3^+) groups, calculated with Rosetta, are indicated in brackets (red). Measured distances between NEMO Lys residues and IKKβ F734, T735, and A736 are shown in Figure S2.

The analysis of Lys(ε-NH_3^+) pK_a, performed with the Rosetta software, led to pK_a values of 11.4, 9.9 and 10.4 for NEMO K90, K96 and K102, respectively (shown in Figure 2), indicating that K96 may be the most nucleophilic site of the series. This residue, together with K90, is particularly close to IKKβ NBD (the measured distances vary from approximately 7 and 11Å, all details are shown in Figure S2), lying in the surrounding of the peptide *N-terminus*. In this region, the short distance between the amide proton in the backbone of IKKβ F734 and the side

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chain of NEMO E89 indicates the formation of an intermolecular H bond (Figure S2). In addition, F734 shows hydrophobic interactions with NEMO L93. As these non-covalent forces conceivably play a significant role in the complex stabilization, we included the F734 residues as the peptides' *N-termini*, resulting in the final FTALDWSWLQTE sequence. Finally, moving towards the peptide *C-terminus*, the hydrophobic features and the peculiar conformation of the WSWL fragment appear crucial for the PPI stabilization. In light of these observations, the three *N*-terminal residues of the FTALDWSWLQTE sequence (i.e., IKKβ residues F734, T735 and A736) were identified as potential anchoring points for the SA tag, ideally aimed at the engagement of either K90 or K96, without disrupting the relevant non-covalent interactions of the wild-type sequence (Figure 3A). As a final feature of the target peptides, a β-alanine unit was located at the *N-termini* as a spacer for the peptides labelling with fluorescein isothiocyanate (FITC), an essential dye for fluorescent anisotropy (FA) studies.

The designed peptide sequences are represented in Figure 3A, and include the wild-type peptide **4** and its three analogues with single-point mutations at *N*-terminal F, T and A residues with Pra (peptides **5**, **6** and **7**, respectively). These peptides were prepared by automated, microwave-assisted SPPS, an efficient technique for peptide production within a short period of time.[16] Following the HPLC purification of Pra-bearing peptides **5-7**, the alkyne group of each compound was easily connected to either $\mathbf{SA_O}$ and $\mathbf{SA_C}$ azides through CuAAC, to give the corresponding SA-tagged peptides. All compounds were purified by HPLC and the chromatograms of crude reaction mixtures (see Figure S3 in the Supplementary Information) indicated quantitative conversions of all alkynes **5-7** into the corresponding triazoles. Isolated products were characterized by LC-MS, as detailed in the Supplementary Information file.

Following a published protocol,[13b] FA assays were performed using recombinant NEMO, tagged with glutathione-S-transferase (GST) and produced by a commercial supplier. The resulting peptide (compound **4** in Figure 3A) showed low affinity for GST-NEMO (K_d > 25 μM, as no FA saturation was observed at high protein concentrations, see Figure 3B), in line with data reported elsewhere for NBP.[17] At this stage, the six SA derivatives were screened in parallel against GST-NEMO in FA binding studies, and the results are shown in Figure 3C. Among these compounds, $\mathbf{6\text{-}SA_C}$, $\mathbf{7\text{-}SA_C}$ and $\mathbf{7\text{-}SA_O}$ showed the best binding performances, with saturation of FA values over 1 μM concentration. Of note, we observed no significant differences among FA data recorded at various incubation times. For homogeneity, all data described here were obtained upon 30 minutes of incubation.

This screening prompted us to investigate the affinity of these hit compounds in comparison with their cognate alkyne precursors **6** and **7**. The results of this experiment, performed in duplicate, are shown in Figure 3D, unveiling a remarkably higher binding affinity of SA derivatives compared to the original Pra-modified peptides. Compound $\mathbf{6\text{-}SA_C}$ proved particularly promising, with an apparent K_d value of 60 ± 9 nM. Finally, to verify the impact of the peculiar SA reactivity on the observed affinity, the alkyne group of peptide **6** was reacted with the azide unit of phenol **3**, devoid of the formyl group. This led to an analogue of lead compound $\mathbf{6\text{-}SA_C}$ (named **6-3**), with suppressed aminophilic behaviour. In a dedicated FA study, including peptide **6** and its derivatives $\mathbf{6\text{-}SA_C}$ and **6-3**, the absence of the aldehyde proved detrimental for the adhesion properties of the peptide (Figure 3E), thus confirming the importance of the SA reactivity for the peptide binding potency.

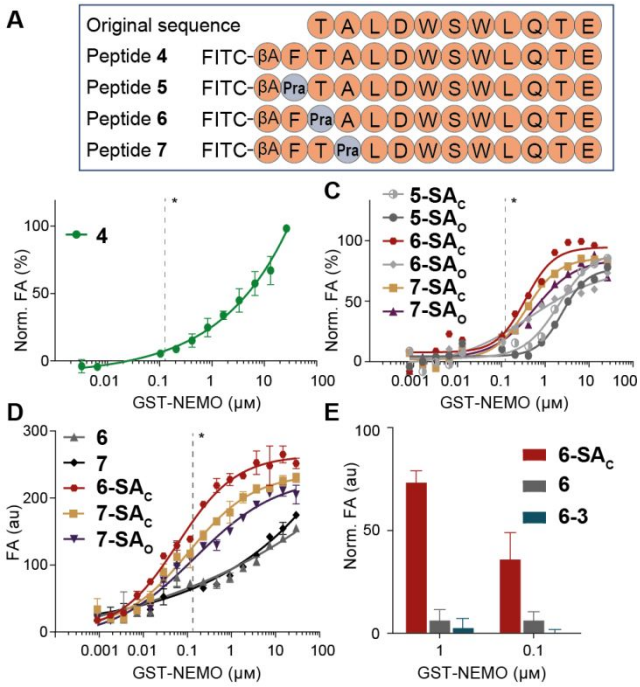

Figure 3. A) design of modified NEMO-binding peptides with the Pra residue inserted in place of N-terminal phenylalanine (F), threonine (T) or alanine (A) residues. Peptides **4-7** were equipped with a β-alanine (βA) spacer and fluorescein isothiocyanate (FITC) for FA binding assays. **B-D**) FA data of synthesized peptides and their SA derivatives, incubated in PBS (pH 7.4) at 125 nM concentration with GST-NEMO at variable concentrations. *The dotted line marks the GST-NEMO concentration of 125 nM, equimolar to the ligand in solution: K_D values similar or lower to this threshold are named herein as "apparent K_D". **E**) FA data of alkyne **6**, $\mathbf{6\text{-}SA_C}$, and the aldehyde-free analogue **6-3**, incubated at 125 nM concentration with 1 and 0.1 μM GST-NEMO.

These FA data confirmed our hypothesis that the tailored insertion of the SA tag into a peptide binder can significantly increase its affinity for the protein target. With the present data, we can only presume that the enhanced affinity is due to the formation of an intermolecular imine adduct, as only in-depth investigations through mass spectrometry or X-ray crystallography can ultimately prove this interaction.

At this stage, we focused on the characterization of our peptide's solubility properties, as the renowned solubility issues of peptides can hinder the follow-up investigations, both in cellular and biochemical assays *in vitro*. For this purpose, the aldehyde-bearing peptide $\mathbf{6\text{-}SA_C}$ and its alkyne precursor (**6**) were dissolved in DMSO at various concentrations and then diluted 1:100 in PBS, to give samples at 5 μM, 125 nM, and 10 nM in PBS (+1% DMSO). These samples were then subjected to dynamic light scattering (DLS) studies and the results are shown in Figure S4 (Supplementary Information). In particular, while peptides formed large and stable aggregates at 5 μM

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concentrations, the 125 and 10 nM solutions resulted in a rapid decay of correlograms and a shift of size distributions toward small aggregates. These data indicate the 125 nM concentration (*i.e.*, the value adopted in FA studies) as a solubility limit for these species in solution. As a consequence, future studies involving higher sample concentrations will require structural optimization or a fine tuning of sample preparation protocols (*e.g.*, screening of buffers, surfactants, etc.).

Conclusions

We have described herein a protocol for a general functionalization of peptide ligands with the SA tag, *i.e.*, a well-known amine-engaging electrophile. Ideally, our design aims at the reversible-covalent engagement of amino groups in proteins and the generation of lead peptides with ultra-high affinity. The preliminary insertion of the Pra residue in a restricted area of the peptide's primary sequence was guided by visual inspection of crystal structures available in the PDB, but more systematic approaches are also feasible. The late-stage peptide modification with SA-azide fragments (**SA_O** and **SA_C**) through CuAAC avoids the use of protecting groups and generates the final products with high efficiency (see chromatograms in Figure S3), ideally allowing the application of this strategy in highly diluted peptide solutions (*e.g.*, in combinatorial libraries).

In our NEMO-binding peptides, the increased binding affinity observed for our SA-tagged ligands compared to the control compounds supports the feasibility of SA-tagged "anti-inflammatory peptides". However, the self-assembly tendency of these peptides, revealed by DLS, may limit their efficient cellular uptake and the interaction with the biological target. Therefore, future biological evaluation will consider peptide incorporation in copolymers or the installation of cell-penetrating sequences.

Considering the high abundance of Lys residues in the proteome and their preferential expression on protein surfaces, we believe that the SA insertion into peptide ligands holds promises for the development of synthetic ligands with unprecedented affinities and prolonged residence time on the target.

Author contributions

Conceptualization, Supervision and Writing: RB, ADC; Methodology, Investigation and Data Curation: MM, KP, FU, LB, DR, ES; Resources: SS, LP, SP; Funding Acquisition: RB, ADC, ES, SS, LB.

Conflicts of interest

There are no conflicts to declare.

Data availability

The data supporting this article have been included as part of the Supplementary Information.

Acknowledgements

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to the attention of
RSC Chemical Biology Editorial Office

Milan, 31st of October, 2025

Re: **Data Availability Statement**

Dear Editor,

With reference to the manuscript by M. Mason *et al.* titled *"Late-Stage Peptide Modification with Salicylaldehyde Tag Enhances Affinity for Nuclear Factor-kappa B Essential Modulator"*, we hereby declare that **the data supporting this article have been included as part of the Supplementary Information**.

The present statement is also included in the submitted manuscript file.

With best regards,

the corresponding authors,
Prof. Alberto Dal Corso and Dr. Raffaella Bucci

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