The Development of a Fluorescence-Based Competitive Assay Enabled the Discovery of Dimeric Cyclic Peptide Modulators of Ubiquitin Chains

Ganga B. Vamisetti, Roman Meledin, Mickal Nawatha, Hiroaki Suga, and Ashraf Brik*

Abstract: Development of modulators targeting specific interactions of ubiquitin-based conjugates with their partners is a formidable task since it requires a suitable screening assay and homogeneous ubiquitin conjugates. We developed a novel high-throughput strategy for screening ligands for Lys48-linked tetraubiquitin chain in a relatively simple, fast, and affordable manner. This approach combined with a state-of-the-art toolbox of chemical protein synthesis and a specially optimized Cys deprotection protocol enabled us to design highly potent, Lys48-linked tetraubiquitin chain selective "next generation" dimeric peptide modulators. The dimeric peptide exhibited cancer cell permeability and induced cell death with higher efficiency compared to its monocyclic analogue. These features make our dimeric peptide a promising candidate for further studies using in vivo models. Our assay can be adopted for other various ubiquitin chains in their free or anchored forms as well as conjugates for Ub-like modifiers.

Protein–protein interactions (PPIs) regulate numerous essential cellular processes. Exploring such interactions is crucial for understanding cellular events in health and disease and thus may contribute to fundamental research and drug discovery.[1] Among the modulators that are available to target important PPIs, macrocyclic peptides (MCPs), which have gained special attention.[2,3] This is due to their advantageous properties of blocking large surfaces of PPIs, on the one hand and their high stability, cellular permeability, affinity and target selectivity on the other.[4–6] Thus, MCPs, which lay in their physical and pharmacological characteristics between small molecules and protein therapeutics, combine advantages of both drug families.

Development of MCPs with desired properties often involves screening of large libraries, using various approaches.[7–9] Hits are then characterized thermodynamically and kinetically using repertoire of standard biophysical techniques, such as surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC). These well-established approaches provide quantitative output for example, K_{on}, K_{off}, K_d, though require highly expensive instrumentation and appropriate training. Even when available, many of these approaches have certain limitations, including medium/low throughput (for SPR and ITC),[10] imperfect detection of low molecular weight ligands[11] (for SPR), and requirement for large sample quantities (for ITC). Therefore, there is need to develop alternative strategies to enable fast, high-throughput, cheap and easy to implement screening of peptide libraries.

One useful alternative is a competitive binding strategy, which involves competition of labeled and unlabeled ligands for the available binding site on the target. Notably, the obvious requirement for this strategy is a generation of a homogenous target and of a labeled ligand with characterized binding parameters. We envisioned utilization of similar principles to devise a MCP screening platform against K48-linked tetra-ubiquitin (tetra-UbK48) chain. Ubiquitination is a complex posttranslational modification, involved in proteasomal degradation of the majority of proteins and hence in the regulation of various cellular processes.[12,13] Therefore, the development of potent and selective agents for its targeting is important to study crucial biochemical events and to discover drug candidates.[14] Since tetra-UbK48 chain is not a direct product of translational machinery, but rather post-translationally assembled complex conjugate, its generation in completely homogenous form by conventional enzymatic approaches is challenging. Here we report on the development of a novel competitive fluorescence high-throughput strategy for screening ligands for Lys48-linked tetraubiquitin chain. This approach enabled us to design highly potent, Lys48-linked tetraubiquitin chain selective "next generation" dimeric peptide modulators. The dimeric peptide exhibited cancer cells permeability and induced cell death with higher efficiency compared to its monocyclic analogue.

We have previously reported various synthetic strategies for the total chemical synthesis of several polyUb chains in their free or anchored forms to protein targets,[13] which enabled exploration of important biological processes.[15,16] Thanks to these achievements, we have recently developed **Ub4_ix** mono-cyclic peptide, which selectively binds tetra-UbK48 chains with high affinity, leading to early- and late stage apoptosis in cells.[17,18] To further advance the discovery of next generation cyclic peptides, we aimed to develop a competitive-based assay to screen peptide libraries for tetra-

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UbK48 binding. We envisioned our assay to proceed through the following four main steps, each terminated by washings with a binding buffer. First, biotin tagged tetra-UbK48 will be immobilized in a constant amount (≈ 1 µg per well) in streptavidin covered 96-well plate (Figure 1(i and ii)). Then, the unlabeled peptides will be incubated in excess in each well to saturate binding to the target. The unlabeled peptide-standard (**Ub4_ix**) with known binding parameters will be also introduced in one of the wells for the subsequent calculation of relative affinity. Next, fluorescein-5-maleimide (FITC) labeled peptide-standard (**Ub4_ix-FITC**) will be introduced in an equal molar ratio to the target (tetra-Ub) in order to compete with the unlabeled peptides. Finally, the bound peptides will be released from wells by treatment with 6 M guanidine hydrochloride solution followed by measuring the gained fluorescence (Figure 1 (i)).

The obtained values are then compared to the fluorescence of the control well containing the unlabeled peptide to calculate the relative binding. The gained fluorescence should be inversely proportional to the binding affinity of the peptide candidate. In addition to measuring the relative binding, our protocol could be also slightly modified to determine the dissociation constant (K_d) of the labeled peptides. For this purpose, after the immobilization step of tetra-Ub, different concentrations of the labeled peptides must be incubated, followed by release with 6 M guanidine hydrochloride and fluorescence measurement (Figure 1 (ii))

To test our assay, we first prepared fluorescently labeled our recently developed cyclic peptide **Ub4_ix**,[17] which was prepared by SPPS (SI, Figure 19) and applied our above describe steps to determine K_d values with the unlabeled peptide. The synthesis of this peptide was carried according to Scheme 1 A. We then measured the fluorescence as a function on increasing concentration of the peptide to plot the binding curve and calculate the dissociation constant (Figure 1 G). Notably, K_d of **Ub4_ix-FITC** measured by our assay is in the range of that of **Ub4_ix** measured by SPR[17] (35.09 ± 2.66 vs. 6.00 ± 1.00 nM, respectively), which indicates the utility of the method.

We then applied our screening method for the development of peptides with improved binding to tetra-UbK48. For this, we first performed an Ala scan on the **Ub4_ix** to shed light on the contribution of each residue in sequence and perhaps further modify the sequence to increase its binding affinity (Figure 2 and SI Figure 2). We found that the analogue **Ub4_ix_S7A** exhibited slightly improved affinity to tetra-Ub K48, as shown by the $\approx 13\%$ decrease in the relative fluorescence compared to **Ub4_ix**. After an additional diversification at position "7" in our original scaffold, we detected that each of the mutants **Ub4_ix_S7W**, **Ub4_ix_S7Y**, **Ub4_ix_S7D** (Scheme 1 A), and especially **Ub4_ix_S7C**, **14**, (Scheme 1 B) further improved the binding parameters (20–40%) (Figure 2).

Notably, Cys is a unique residue with its exceptional nucleophilic character of its thiol side chain, which makes it useful for late-stage selective modifications. Therefore, we took advantage of the observation of incorporating Cys without abolishing the affinity at position "7" for further modifications with a range of small molecules, which might
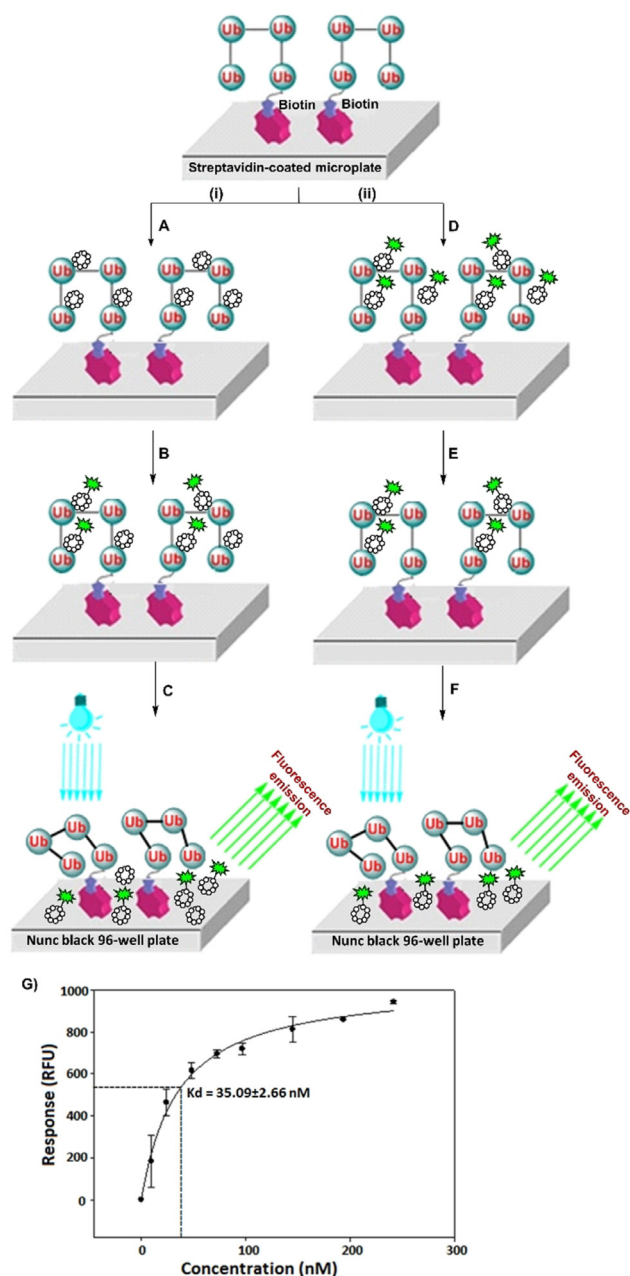

Figure 1. Principle of our competitive tetra-Ub binding assay. i) Schematic presentation of the screening of various unlabeled peptides. A) An unlabeled cyclic peptide is introduced for binding to the biotin-tetra-Ub. B) A labeled cyclic peptide is introduced to compete with the binding of the unlabeled peptides to the biotin-tetra-Ub followed by washing the unbounded peptides. C) The bound peptides are released using 6 M Gn·HCl and the fluorescence intensity is measured. ii) Schematic presentation of the measurement of the dissociation constant (K_d) of FITC-labeled peptides with different concentrations. D) A FITC-labeled cyclic peptide with different concentrations is introduced in different wells for binding to the biotin-tetra-Ub. E) The unbounded labeled cyclic peptide is washed out. F) The bound peptides are released using 6 M Gn·HCl and the fluorescence intensity is measured. G) Binding curve of FITC-labeled **Ub4_ix** to tetra-Ub K48. The data points were fitted using an equation representing a one site non-cooperative ligand binding model $Y = Bmax*X/(K_d + X)$, where X is ligand (**Ub4_ix-FITC**) concentration, Y: specific ligand binding, Bmax: maximum specific ligand binding, and K_d: dissociation constant. The K_d value of 35.09 ± 2.66 nM was determined. All measurements were performed in triplicate.
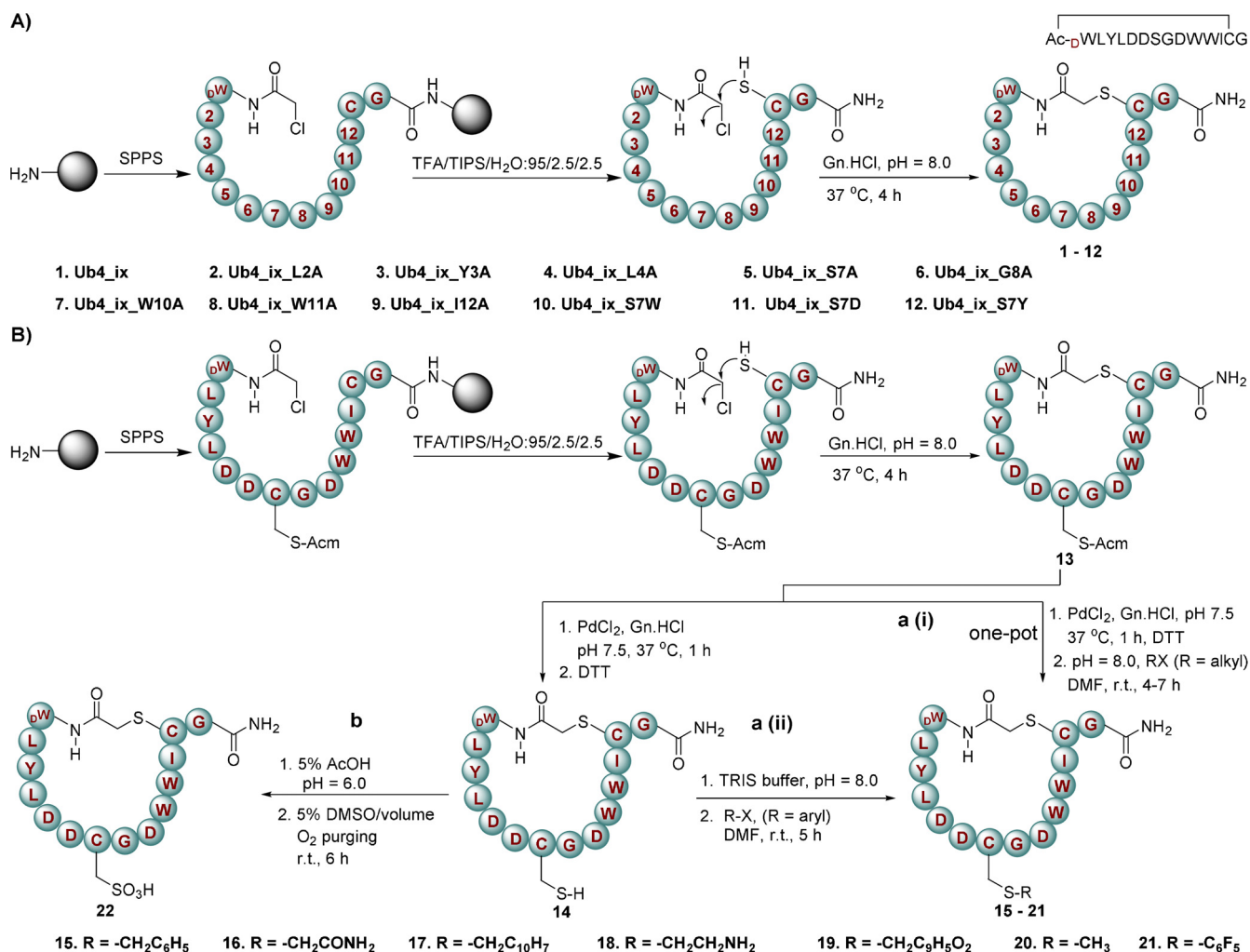
Scheme 1. General schematic presentation of the synthesis of thioether-linked cyclic peptides. A) Ala scan and mutation at the 7th position. B) Schematic presentation of the modification of Cys7.
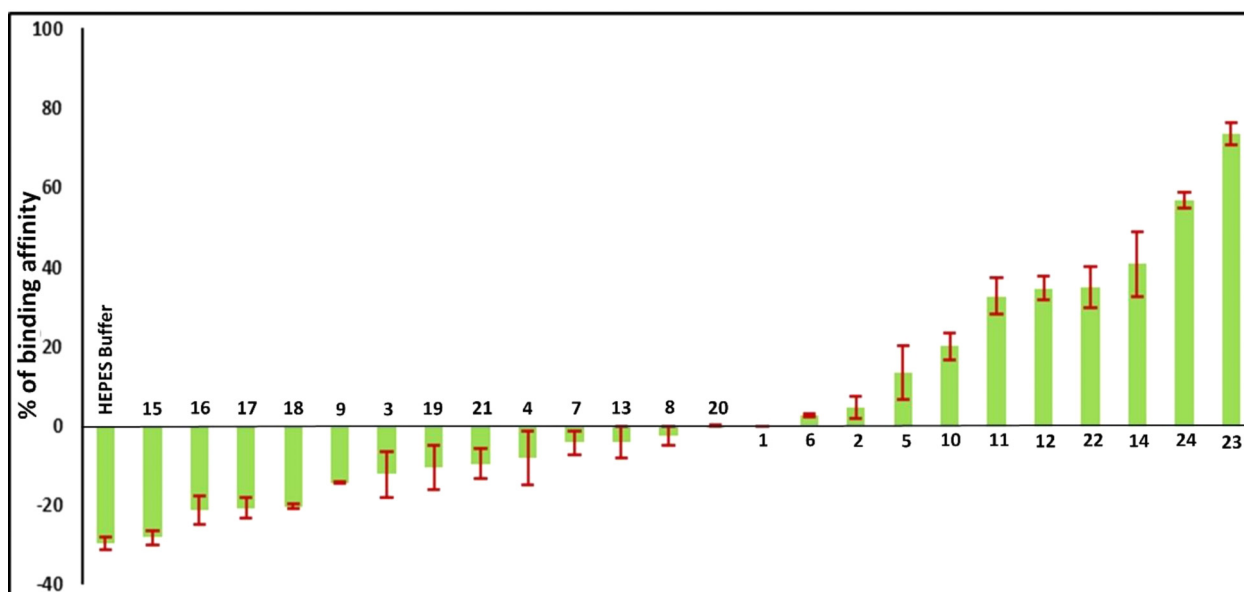

Figure 2. Binding of various thioether-linked cyclic peptides to tetra-Ub K48, normalized to the affinity of **Ub4_ix**. HEPES buffer (50 mM HEPES, 150 mM NaCl, 0.1% Tween, pH 7.3; Negative control). All measurements were performed in triplicate and in at least three biological replicates. Error bars represent standard error.

increase the affinity of the peptide. A series of analogues were prepared using selective thiol alkylation, arylation and oxidation chemistries (Scheme 1B and see Section 5 in SI). Most of the modified peptides, however, showed unaffected or abolished binding, compared to the initial **Ub4_ix** scaffold.

With the knowledge that dimeric peptides could possess favorable properties compared to their monocyclic analogues[19] due to their bivalent binding mode to the target,[19] we decided to test this rationale on our system by preparing a disulfide bond containing dimeric peptide **23**, **(Ub4_ix_S7C)₂** (Scheme 2a). Pleasantly, **23** displayed a much higher affinity for the target ($\approx 70\%$ change in the relative signal). Notably, disulfide bond containing peptides may show limited applicability in cellular studies, due to the cellular reducing environment. To overcome this potential-limitation, we designed a dimeric peptide analogue **24**, **(Ub4_ix_S7C)₂CH₂**, bearing a stable thioether-based linker (Scheme 2b).

The synthetic route for the preparation of dimeric analogues and especially of **(Ub4_ix_S7C)₂CH₂-FITC** labeled stapled dimer was not straightforward and required particular attention. To achieve the synthesis of the unlabeled dimers,

orthogonally acetamidomethyl (Acm) protected Cys7 was incorporated to the scaffold, followed by cleavage from the resin and selective cyclization to give the thioether linked cyclic peptide **13** (Scheme 1B). Subsequently, Acm protecting group was removed by treatment with 10 equiv PdCl₂,[20,21] followed by quenching the reaction with 40 equiv of dithiothreitol (DTT) at room temperature to give peptide **14**, **Ub4_ix_S7C**, in 42% isolated yield (Scheme 1B). Finally, **14** was dissolved in AcOH:H₂O (4:1) followed by the addition of 2 equiv of I₂ at 37°C for overnight to form **23** in 23% yield (Scheme 2a). Alternatively, **14** was dissolved in H₂O:THF (9:1) followed by addition of diiodomethane under basic conditions at room temperature for 48 h (Scheme 2b) to give the **24** in 18% yield.

In order to achieve FITC-labeled stapled dimer, both Cys residues had to be orthogonally protected by different protecting groups to enable selective unmasking at a later stage of synthesis. For this aim, we used Acm and tert-butylthiol (-SᵗBu) thiol protecting groups. We prepared peptide **25** using SPPS followed by cleavage and selective cyclization to give the thioether linked cyclic peptide **26** (Scheme 3a). Next, the -SᵗBu protecting group was orthog-
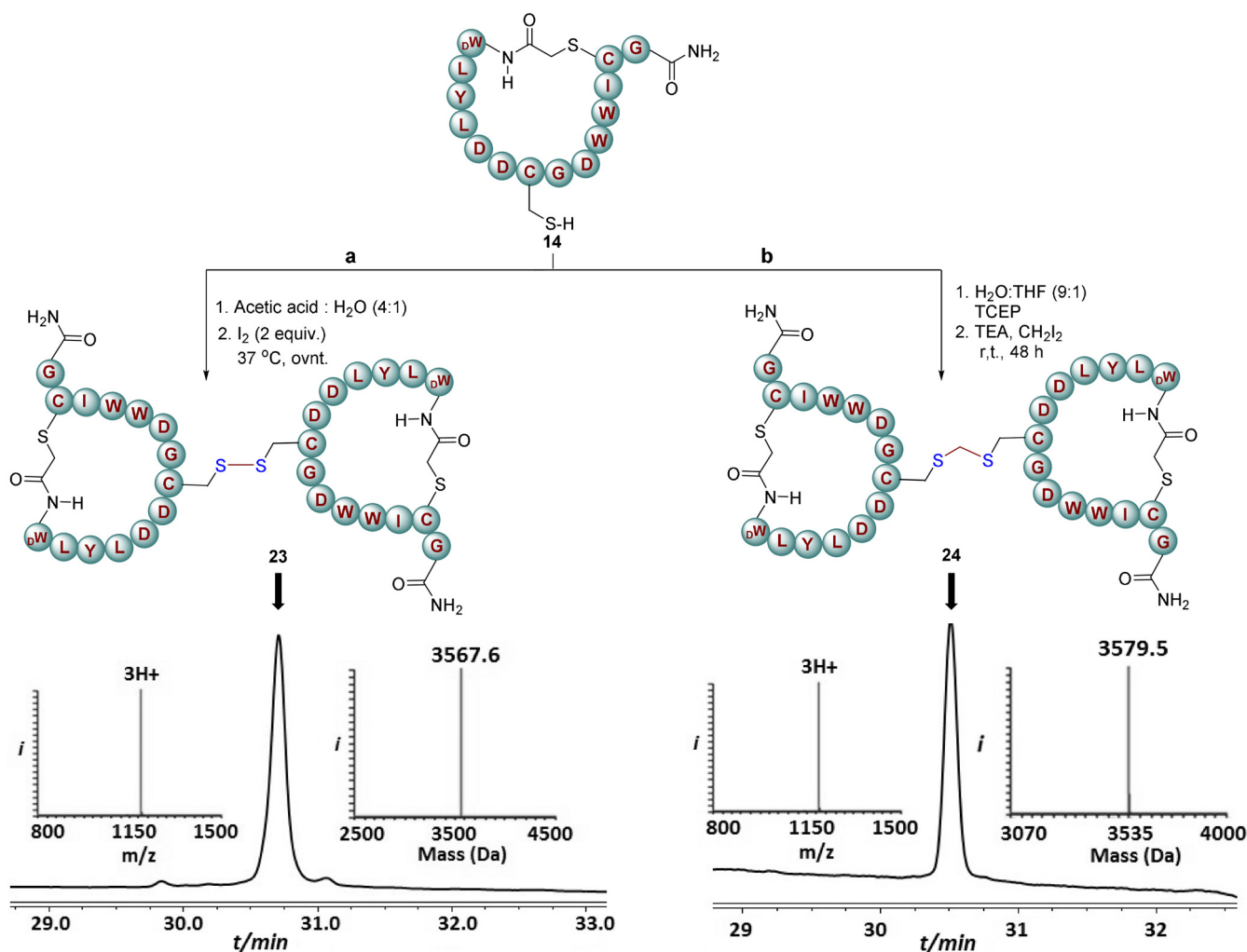
Scheme 2. Synthesis of dimeric cyclic peptides. a) Dimer formation through disulfide bond formation (Observed mass: 3567.6 ± 0.4 Da, calcd: 3568.0 Da, average isotopes). b) Dimer formation through -S-CH₂-S- bridge (Observed mass: 3579.5 ± 0.4 Da, calcd: 3579.9 Da, average isotopes).
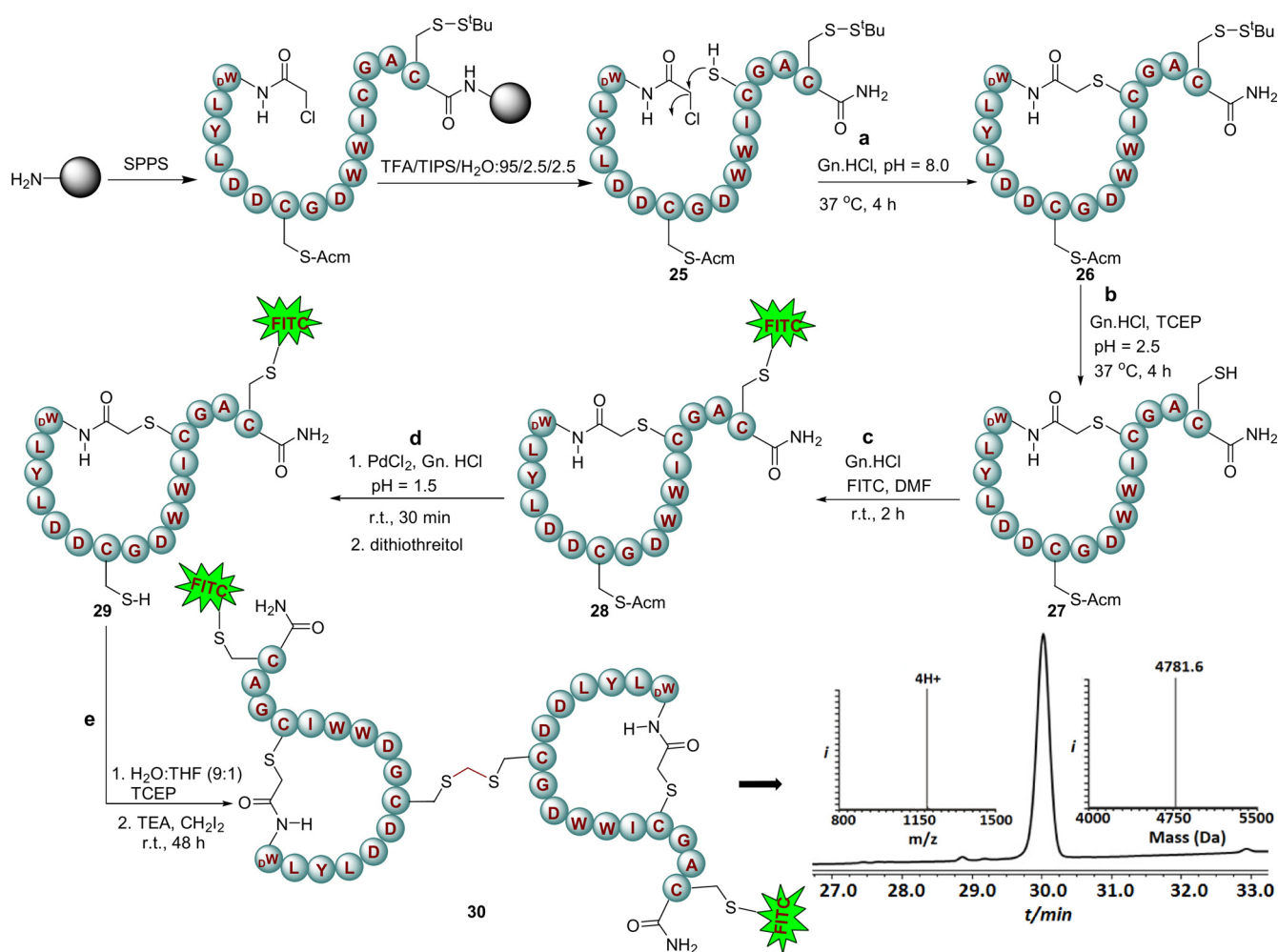
Scheme 3. Synthesis of **(Ub4_ix_S7C)$_2$CH$_2$-FITC** (Observed mass: 4781.6 ± 0.1 Da, calcd: 4782.4 Da, average isotopes). FITC = Fluorescein-5-maleimide.

onally removed through selective reduction of a disulfide bond by treatment with tris-(2-carboxyethyl) phosphine (TCEP) in 6 M Gn·HCl pH 2 at 37 °C for 4 h to give **27** (Scheme 3b) in 42% yield.[22] Subsequently, 2 mM of **27** dissolved in 6 M Gn·HCl (pH 7.5) was treated with 3 equiv of fluorescein-5-maleimide in DMF at room temperature for 2 h to give FITC labeled peptide **28** (Scheme 3c) in 40% isolated yield. In the following step, we attempted to selectively remove the Acm protecting group using our previously developed conditions.[20] Here, however, under these conditions, we observed disruption of the FITC moiety. To overcome this, we examined a range of different loadings of PdCl$_2$ at two different temperatures (37 °C and r.t.) under two different pH values (7.5 and 1.5) and for several periods (5, 10, 15, 30, and 45 min). We found that treatment of **28** with 2 equiv of PdCl$_2$, pH 1.5 at room temperature for 30 min (SI, Sections 11 and 12) gave the best results for the selective removal of Acm without disruption of FITC, and offered peptide **29** (Scheme 3d) in 20% isolated yield. Finally, a FITC labeled stapled dimer **30**, **(Ub4_ix_S7C)$_2$CH$_2$-FITC**, was prepared in 12% yield in a similar fashion to described above (Scheme 3e).

We then applied our assay with the FITC-labeled stapled dimer **30** to evaluate the dissociation constants our stapled dimer **24**. The K_d was determined to 12.32 ± 1.26 nM, which is threefold better than the monomer (SI, Figure 22). Notably the stable dimer analogue showed slightly decrease in binding compared to the disulfide containing peptide **23**.

An important and desired property of this dimeric peptide is its ability to enter live cells without additional manipulations. We investigated the cellular delivery of **30**, **(Ub4_ix_S7C)$_2$CH$_2$-FITC** to HeLa cells using confocal microscopy. We observed permeability and cytosolic localization of the peptide detected by FITC signal within 2 h after incubation, (SI, Figure 24). Finally, we assessed the cellular effect of **(Ub4_ix_S7C)$_2$CH$_2$** by conducting a viability assay with annexin V-FITC apoptosis/necrosis detection kit (BD Biosciences) using flow cytometry. Treatments with the proteasome inhibitor MG132 and DMSO were performed as positive and negative controls, respectively (SI, Figure 23). Pleasantly, **(Ub4_ix_S7C)$_2$CH$_2$** was able to induce cell death in Hela cells after 24 and 48 h incubation in a dose- and time-dependent manner. Furthermore, **(Ub4_ix_S7C)$_2$CH$_2$** showed increased early- and late stage apoptosis compared to our
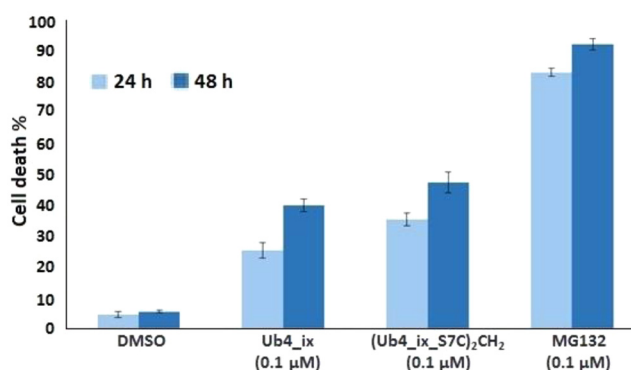
Figure 3. Cytotoxicity of the cyclic peptides in HeLa cells. Cells were exposed for 24 and 48 h to the indicated **(Ub4_ix_S7C)₂CH₂** and **Ub4_ix** peptide or MG132. Cell viability was measured by FACS (Bars represent mean values).

first-generation peptide **Ub4_ix**, which correlates with its improved potency (Figure 3 and SI Figure 23).

In summary, we have developed a high-throughput, simple, and affordable, fluorescence-based competitive assay for screening of peptides against tetra-UbK48. We applied our assay to engineer dimeric peptides with an improved affinity compared to their first-generation analogue. The generation of labeled dimeric peptides required special conditions for stepwise orthogonal deprotection strategy and especially employed modified conditions for selective Cys(Acm) deprotection in the presence of FITC. Our developed dimeric peptide exhibited cell permeability, despite the size difference with the monomeric peptide, and exhibited slightly improved activity. By introducing various chemical modifications like attachment of cationic or hydrophobic tags or changing the linker's nature we might be able to further improve the permeability of the dimeric peptide which will be translated to enhanced biological activity. Also, induced cell death compared with the first-generation monocyclic analogue **Ub4_ix**. This highlight the added value of polycyclic peptides, which are crucial for a molecule to be considered as a drug candidate.[23] In addition, our labeled dimeric peptide **30** can potentially be used as a polyUbK48-specific staining agent with antibody-like properties, where live cells would be visualized without a need for permeabilization. This, in turn, will enable exploration of Ub chain dynamics in real-time conditions. Future work in our lab will examine this tethering approach with the second generation monocyclic peptides[18] as well as the optimal length of the linker to achieve better properties of the dimer. A similar strategy could be applied for other important and complex targets, including hybrid Ub chains[24,25] and modified histones.[26]

Acknowledgements

Conflict of interest

The authors declare no conflict of interest.

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