Conjugation of Spermine Enhances Cellular Uptake of the Stapled Peptide-Based Inhibitors of p53-Mdm2 Interaction

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Abstract

We report the first synthesis of the *C*-terminally spermine-conjugated stapled peptide-based inhibitors of the p53-Mdm2 interaction. Subsequent biological, biophysical and cellular uptake assays with the spermine-conjugated stapled peptides revealed that spermine conjugation minimally affects biological activity while significantly increases peptide helicity and cellular uptake without apparent cytotoxicity.

Keywords

spermine; peptides; helical structure; protein-protein interaction; cellular uptake

Peptide helices are found frequently at the interfaces of protein-protein interactions that mediate all facets of cellular processes including cell cycle control and apoptosis.[1] As a result, there has been a growing interest recently aimed at translating these helical peptides into biological therapies targeting the intracellular protein-protein interactions.[2] Since short peptides exhibit poor pharmaceutical properties, i.e., poor cell permeability and low metabolic stability, numerous strategies have been developed to increase their proteolytic stability as well as their cellular uptake. Among them, selective cross-linking of peptide side-chains (also referred to as peptide 'stapling') via selective chemical reactions, e.g., disulfide bond formation,[3] olefin cross metathesis,[4] lactam formation,[5] and 1,3-dipolar cycloaddition reactions,[6] has emerged to be one of the most promising approaches.[7]

Recently, we reported the discovery of a distance-matching cross-linker, 6,6′-bisbromomethyl-[3,3′]bipyridine (Bpy), that reacts with a pair of cysteines located at *i,i*+7 positions of a short helical peptide, resulting in a significant enhancement in cell permeability.[8] While it is known that attachment of the positively charged groups to peptides generally leads to enhanced cellular uptake, most studies in the literature have focused on either direct fusion of polyarginine-like sequences[9] such as TAT sequence or substitution of arginines at strategically selected non-continuous sites on the peptides.[10] Herein, we report the first synthesis of polyamine-conjugated, stapled peptides and the

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characterization of the effect of polyamine conjugation on peptide bioactivity, helicity, and cell permeability.

Polyamines such as spermine are positively charged at the physiological pH, and have been used through conjugation as an intracellular delivery aid for the lipophilic small-molecule drugs[11] as well as the negatively charged siRNA.[12] However, the effect of polyamine conjugation to synthetic peptides on cellular transport of peptides has not been reported.[13] According to helical dipole model,[14] placement of the positive-charged capping groups at the carboxyl termini of short helical peptides leads to increased helicity due to the favourable charge-dipole interactions.[15] To probe whether this model is operative in the case of short stapled peptides, we conjugated spermine at the C-terminus of a dicysteine-containing peptide inhibitor of p53–Mdm2 interaction (sequence = LTFCHYWAQLCS), which was used previously as a model in our peptide stapling studies.[8] Scheme 1 shows a representative synthesis of the spermine-conjugated, stapled peptide **4** in seven steps based on a solid phase procedure. Briefly, spermine was first immobilized onto solid support by treating 2-chlorotrityl chloride resin with excess amount of spermine in dichloromethane for 30 min. After extensive washing, selective protection of the secondary amines with Boc was achieved by first masking the terminal primary amine with 2-acetyldimedone[16] to form the *N*-[1,(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl (Dde) derivative followed by treatment with di-*tert*-butyl dicarbonate. The Dde-protecting group was selectively removed by treating the resin with 2% hydrazine in DMF for 15 min. With the spermine-functionalized resin in hand, standard Fmoc-based solid phase peptide synthesis was performed and the linear cysteine-containing peptide was released from the resin after treatment with a TFA-containing cleavage cocktail. After HPLC purification, the fully deprotected linear peptide was incubated with 1.5 equiv of Bpy in a mixed acetonitrile/30 mM NH_4HCO_3 solution at pH 8.5. HPLC analysis of the crude product indicated that the stapling reaction of the spermine-conjugated peptide proceeded with greater than 95% conversion after 1.5 hours (Figure S1). Using this route, we also prepared spermine-conjugated, D,L-dicysteine-stapled peptide **8** because the D,L-dicysteine-stapled peptides have shown greater inhibitory activity towards Mdm2 in our previous work.[8]

To gauge how spermine conjugation affects stapled peptide's biological activity, we determined the inhibitory activities of the spermine-conjugated cross-linked peptides with ELISA and compared them to other stapled peptides reported previously[8] (Figure S2 and Table 1). Interestingly, whereas substitution of two residues at solvent-exposed side of the helix with arginines (His5→Arg and Gln9→Arg) led to addition of +2 charge accompanied by substantial drop (6~20-fold) in inhibitory activity[8] (compare **3** to **2**; **7** to **6**), spermine conjugation resulted in addition of +3 charge and significantly smaller reduction in inhibitory activity (compare **4** to **2**; **8** to **6**). Therefore, spermine conjugation appears to provide a less disruptive route to the addition of net positive charge.

To examine how spermine conjugation affects cellular uptake, we synthesized the *N*-terminal fluorescein-labeled, spermine conjugated, stapled peptides **4** and **8** (Fluo-**4** and -**8**, Table S1) and compared their uptake into HeLa cells as measured by fluorescence activated cell sorting (FACS) to the previously reported fluorescein-labeled stapled peptides[8] (Fluo-**1**–**3** and Fluo-**5**–**7**). As shown in Figure 1, peptide stapling significantly enhanced the cellular uptake (compare Fluo-**2**–**4** to -**1**; Fluo-**6**–**8** to -**5**). Among the stapled peptides, peptides carrying the positive charges (Fluo-**3**, -**4**, -**7**, and -**8**) showed significantly higher uptake than the charge-neutral peptides (Fluo-**2** and -**6**); the spermine-conjugated stapled peptides Fluo-**4** and -**8** exhibited the highest uptake, yielding 16- and 22-fold increase, respectively, over their charge-neutral linear counterparts (compare Fluo-**4** to -**1**; Fluo-**8** to -**5**), indicating that spermine conjugation acts in concert with peptide stapling to further enhance the cellular uptake. The higher uptake observed for the spermine conjugated, stapled peptides Fluo-**4**

and -**8** relative to the arginine-substituted, stapled peptides Fluo-**3** and -**7** could also be attributed to the localized positive charge at the *C*-termini of Fluo-**4** and -**8** versus the dispersed positive charge in Fluo-**3** and -**7**.

To probe structural changes induced by spermine conjugation, we determined the percent helicity of all stapled peptides with far-UV CD measurement (Figure 2). While cysteine cross-linking invariably led to modest increases in percent helicity (compare **2–4** to **1; 6–8** to **5**), the spermine-conjugated stapled peptides showed additional 50% increases in percent helicity than the arginine-substituted stapled peptides (compare **4** to **3**; **8** to **7**). This enhancement in helicity is consistent with the helical dipole model[15] in which the *C*-terminally conjugated, positive-charged spermine stabilizes helical dipole by forming additional hydrogen bonds with *C*-terminal unpaired carbonyl groups. A correlation between percent helicity and cell permeability appears to exist as peptides with higher percent helicity invariably exhibited higher cellular uptake.

To corroborate the FACS results on cellular uptake, a confocal fluorescent microscopy study was performed. We found that while the positive-charged stapled peptides showed stronger intracellular fluorescence than the charge-neutral ones, Fluo-**3** and -**7** treated cells produced significant amount of cellular debris which was absent in Fluo-**4** and -**8** treated cells (Figure S3). This difference in apparent cytotoxicity was verified by ATP assay. Cells treated with the spermine-conjugated stapled peptides (**4** and **8**) were found to be completely viable whereas cells treated with the arginine-substituted peptides **3** and **7** showed 50% and 30% decrease in viability, respectively (Figure 3). The cytotoxicity of **3** and **7** could be attributed to the non-specific cell membrane insertion activity of amphipathic arginine-containing peptide helices with one side of the helix to be hydrophobic and the opposite side to be cationic.[17] In contrast, the devoid of cytotoxicity for the spermine-conjugated stapled peptides could be attributed to their unique mode of helix stabilization. The possible mechanism for uptake of the spermine-conjugated stapled peptides was then probed with a fluorescence-based subcellular localization study (Figure 4). Fluo-**4** showed a more diffusive intracellular distribution, with some overlap with Alexa Fluor 586 labeled transferrin, a fluorescent marker for the recycling endosomes (panel d in Figure 4), suggesting a possible endosome-mediated transport process.

In summary, we have demonstrated a small-molecule approach in rendering stapled peptides cationic through the C-terminal spermine conjugation. The attachment can be readily integrated into the solid phase synthesis protocol and compatible with the peptide stapling conditions. Compared to the arginine-substitution approach, spermine conjugation minimally affected biological activity of the stapled peptides while significantly increased peptide helicity. Together with peptide stapling, spermine conjugation acted in concert to further enhance the cellular uptake of the bioactive peptides without apparent cytotoxicity frequently associated with arginine substitution. Inasmuch as the cationic TAT peptide,[9a] spermine conjugation should provide a simple, small-molecule strategy for intracellular delivery of bioactive peptides and other biotherapeutics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References and notes

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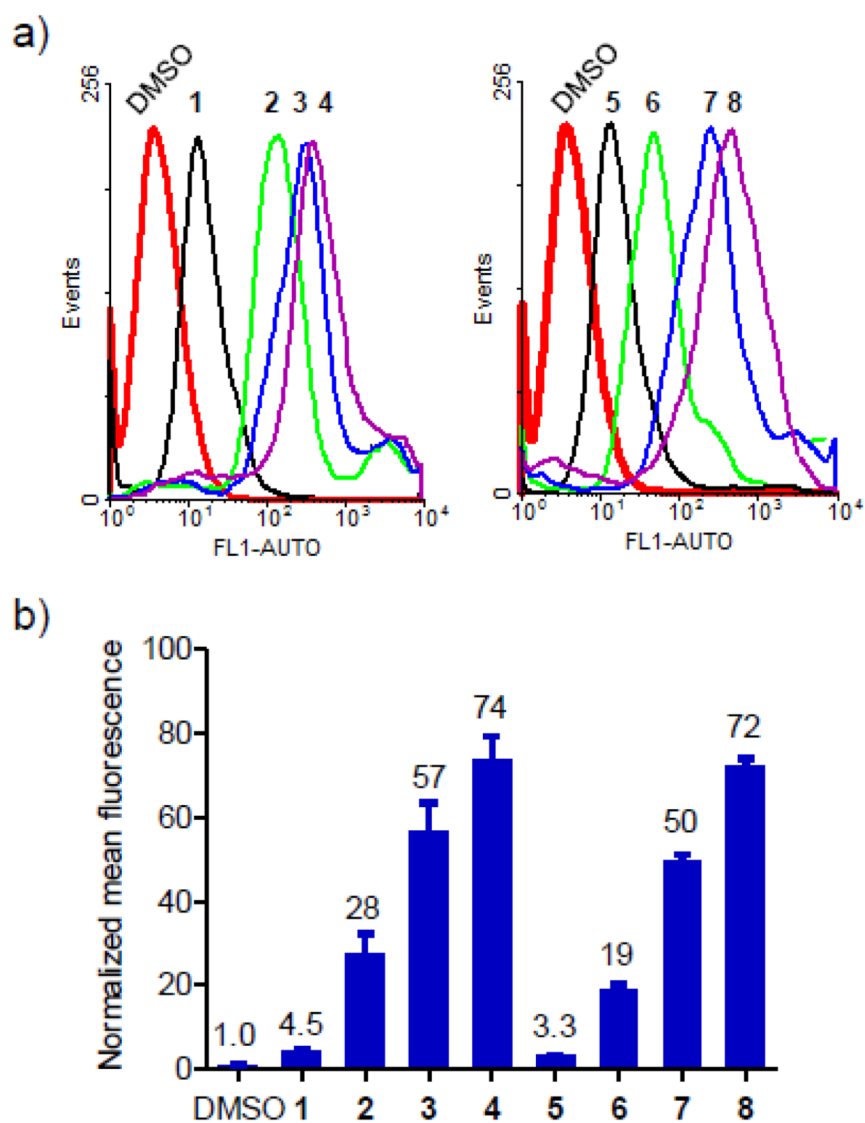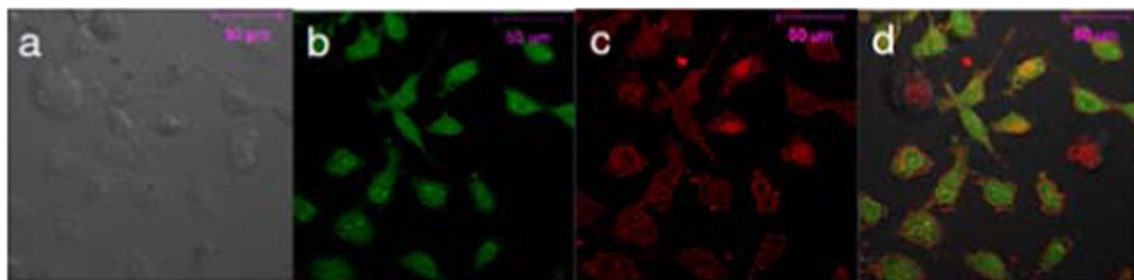
Figure 1.
Flow cytometry analysis of HeLa cells after treatment with 10 μM fluorescein-conjugated peptides, Fluo-**1**–**8**: (a) Representative histograms; (b) Bar graph showing the normalized mean fluorescence. For simplicity, Fluo- prefixes were omitted in the stapled peptide denotations.
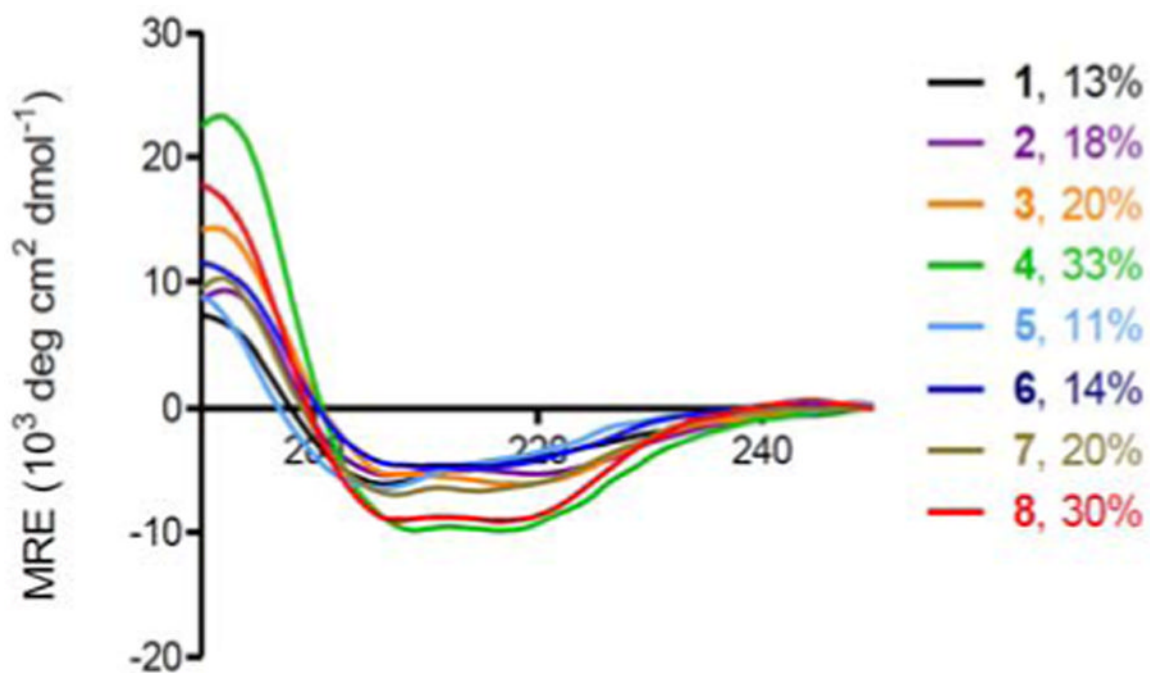
Figure 2.
CD spectra of the linear and Bpy-stapled peptides with or without spermine conjugation and their calculated percent helicity values: The peptides were dissolved in 20% ACN/H_2O for a final concentration of 50 µM. The percent helicity was calculated based on the $[\theta]_{222}$ value.
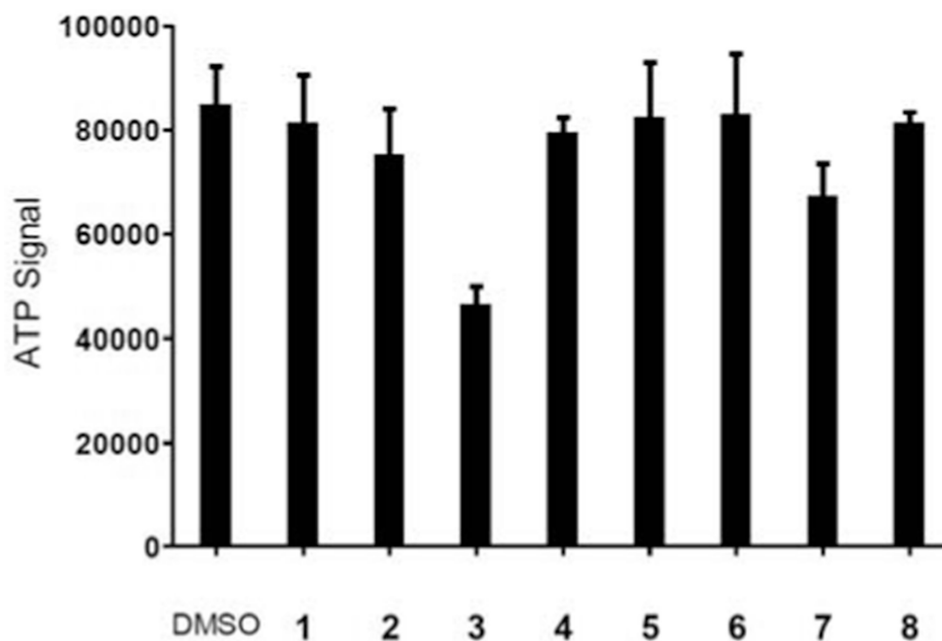
Figure 3.
Spermine conjugation increases cellular uptake of the stapled peptides without apparent cytotoxicity analyzed by ATP assay. The CellTiter-Glo reagent (Promega) was used to measure HeLa cell viability after treatment with 10 µM of the unstapled (**1** and **5**) or the stapled peptides (**2**-**4** and **6**-**8**).

Figure 4.
Confocal fluorescent microscopy of live HeLa cells after treatment with 10 μM of fluorescein-conjugated **4** for 2 hr followed by incubation with 10 μg/mL Alexa Fluor 586 labeled transferrin for 30 min: (a) DIC channel; (b) FITC channel; (c) Fluor 585 channel; (c) merged images of channels a-c.
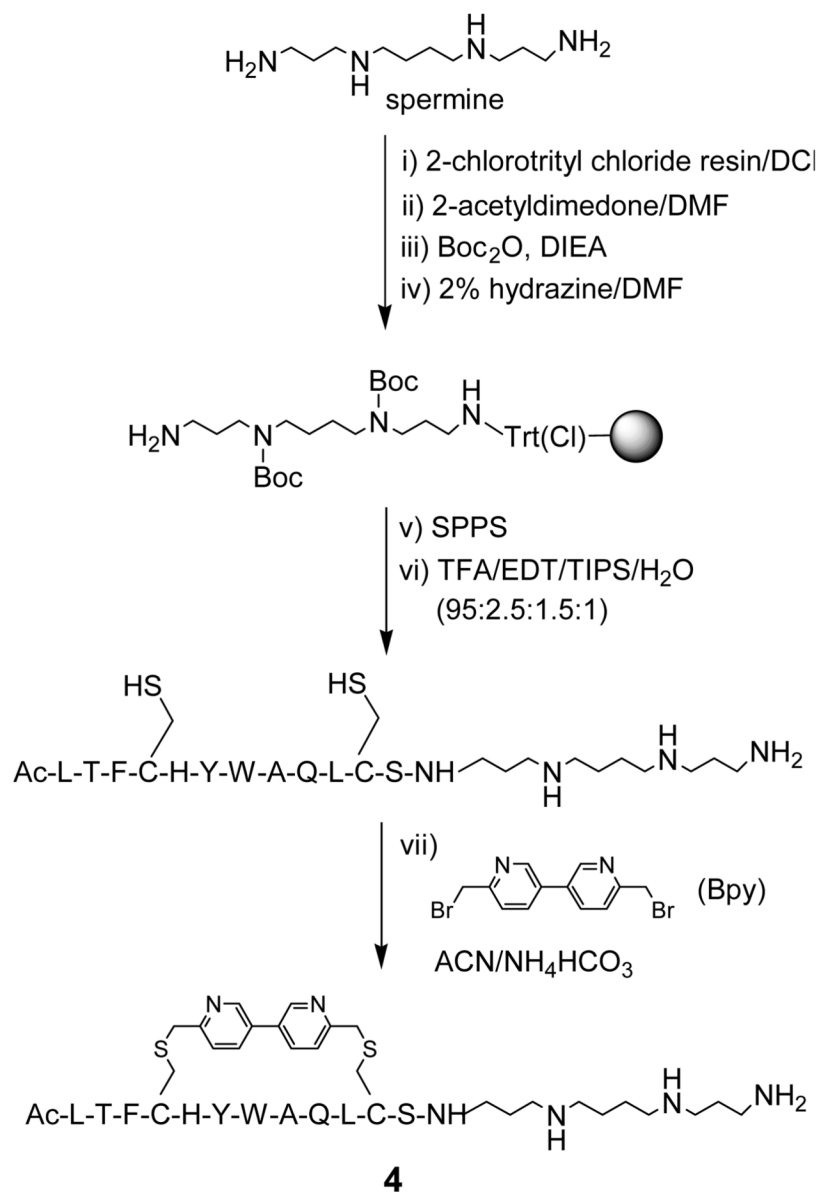
Scheme 1.
Representative synthesis of a spermine-conjugated stapled peptide **4** on solid support.

Table 1

Sequence and Activity of Peptide-Based Inhibitors[a]

peptide	sequence[b]	charge	IC_{50}, nM
1	LTFCHYWAQLCS	0	57 ± 5
2	LTFC′HYWAQLC′S[c]	0	35 ± 5
3	LTFC′**R**YWA**R**LC′S	+2	237 ± 16
4	LTFC′HYWAQLC′S-Sp[d]	+3	46 ± 6
5	LTFcHYWAQLCS[e]	0	13 ± 1
6	LTFc′HYWAQLC′S[f]	0	5 ± 1
7	LTFc′ **R**YWA**R**LC′S	+2	102 ± 8
8	LTFc′HYWAQLC′S-Sp	+3	18 ± 2

[a]ELISA assays were repeated three times to obtain average IC_{50} values and standard deviations.

[b]For non-spermine containing peptides, the *N*-termini were acetylated and the *C*-termini were amidated. For spermine-conjugated peptides, the *N*-termini were acetylated.

[c]C′ denotes Bpy-linked cysteine.

[d]Sp denotes spermine.

[e]c denotes D-cysteine.

[f]c′ denotes Bpy-linked D-cysteine.