Efficient Solid-Phase Synthesis of a Series of Cyclic and Linear Peptoid−Dexamethasone Conjugates for the Cell Permeability Studies

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Cyclic peptides and their cyclic analogs have received a great deal of attention because of their numerous interesting biological activities and their challenging chemical synthesis. It has also been hypothesized that they might improve the cell permeability compared to linear molecules by providing internal hydrogen bonding and generally decreasing the conformational flexibility. In this study, a series of cyclic and linear peptoid−dexamethasone conjugates were rationally designed and efficiently synthesized on solid-phase for systematic cell permeability studies using reporter gene-based assays. These model compounds should be used to reveal how the cell permeability of cyclic molecules is affected by several physicochemical properties, especially, the reduced conformational flexibility and the ring size. In addition, the synthetic strategy that was adopted in this study can also provide a robust platform for postchemical modifications of various molecular scaffolds in solid-phase or solution-phase syntheses.

Introduction

Cyclic peptides are of considerable interest as drug candidates and potential chemical modulators for proteins.[1−6] In some cases, their conformational rigidity may enhance their binding affinity for target proteins while reducing the entropy loss.[7,8] Cyclic peptides often exhibit improved proteolytic resistance compared to their linear isomers.[9,10] The membrane permeability of small molecules is a critical issue in drug discovery or biological applications and has extensively been studied using various in vitro models and computational methods.[11−17] Several physicochemical properties such as the hydrophobicity, molecular size, polar surface area, solubility, desolvation energy, internal/intramolecular hydrogen bonding ability, etc., are potential contributors to the membrane permeability. It has also been suggested that cyclic peptides might improve the cell permeability compared to linear molecules through the elimination of the charged termini, providing internal hydrogen bonding and generally decreasing the conformational flexibility.[18−20] However, careful experimental tests that have been carried out to confirm these suggestions are rare.

Recently, Lokey et al. investigated the membrane permeability of cyclic peptide diastereomers and showed that a cyclic peptide in the study was more permeable than its linear counterpart in a parallel artificial membrane permeability assay (PAMPA).[18,19] They suggested that the formation of conformation-dependent intramolecular hydrogen bonds is critical for passive membrane permeability using PAMPA, in silico prediction, and NMR studies. We also assessed quantitatively the relative cell permeability of cyclic and linear peptides using a reporter gene-based assay, which was developed in the Kodadek's laboratory.[21,22] Interestingly, the cyclic peptides that were employed in the study were not more permeable than their linear counterparts. The cyclic and linear peptides in the study might not have been drastically different in the degree of total intramolecular hydrogen bonding, even though the effects of the internal hydrogen bonding and the ring size of the cyclic peptides on the membrane permeability were not clear because the cyclic peptides were actually decorated with different side chains on the same cyclic peptide scaffold. The importance of the intramolecular hydrogen bonding potential on the membrane permeability was also addressed in the comparison of the relative cell permeability between peptides and peptoids.[23,24] Peptoids were much more cell permeable than their corresponding peptides, suggesting that the peptoids could save the unfavorable desolvation energy of the polar N−H bonds of the peptides. Curiously, it was not clear why the peptides in the study were not less permeable as the size increased from a dimer to an octamer. However, the peptides may have formed intramolecular hydrogen bonds as their size increased, resulting in the reduction of the unfavorable desolvation energy.

In addition to our previous efforts, herein we describe the rational design and the efficient synthesis of a series of cyclic and linear peptoid−dexamethasone conjugates for the systematic cell permeability studies. The permeability studies will be carried out using a reporter gene-based assay in due course to provide significant insight into the physicochemical properties of the cyclic molecules that affect their cell permeability, especially, the reduced conformational flexibility and the ring size.

Results and Discussion

In our previous studies, the relative cell permeabilities of peptides versus peptoids and cyclic peptides versus linear peptides were quantitatively evaluated using a reporter gene-

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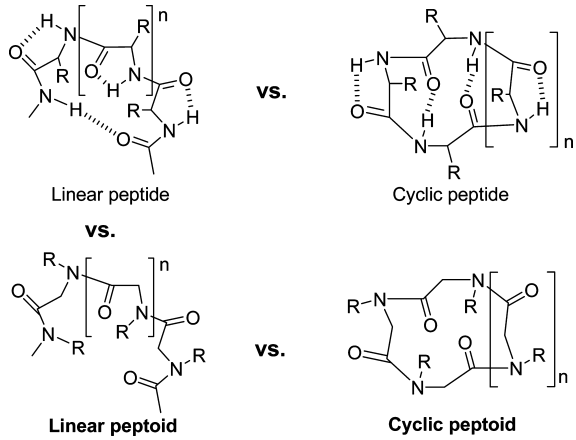


Figure 1. Conformational representation of the model compounds for the membrane permeability.

based assay in vivo, and some useful information was obtained.[21,23] As seen in Figure 1, linear or cyclic peptide models can form intramolecular or internal hydrogen bonds, which are critical to the passive membrane permeability. To further investigate the effects of the reduced conformational flexibility and the ring size of the cyclic molecules on the membrane permeability, we envisioned to examine the relative cell permeability of the cyclic and linear peptoids. Peptoids have many advantages such as easy synthesis,[25,26] resistance to proteolytic activities,[27] improved cell permeability,[23,24] and interesting biological activities.[28−31] We have already determined the optimum cyclization conditions for the synthesis of cyclic peptoids, resulting in the development of cyclic peptoids libraries and microarrays.[32,33] Structurally, peptoids do not contain any polar N−H bonds that can form intramolecular hydrogen bonds. Thus, the linear and cyclic peptoid models were believed to be ideal for the purposes of this study.

At first, a series of cyclic and linear peptoid−dexamethasone conjugates with different sizes were rationally designed (Figure 2). Each series contained four peptoids (**C4**−**C10** and **L4**−**L10**) with alternating 2-methoxyethyl and methyl side chains and three peptoids (**C5**−**C9** and **L5**−**L9**) with different side chains. Initially, both the 2-methoxyethyl and methyl side chains were chosen out of consideration for their water solubility, neutral hydrophobicity and intramolecular hydrogen bonding exclusion. Especially, pairs of the four peptoids (**C4**−**C10** and **L4**−**L10**) in each series should be used to investigate the ring size effect of the cyclic peptoids on the membrane permeability. The other three pairs could be used to confirm if the conclusion that will be obtained using pairs of the four peptoids (**C4**−**C10** and **L4**−**L10**) is a general phenomenon in this study. Peptoid structure would probably contribute to the cell permeability. Actually, peptoids have been reported to form the secondary and even more complex structural features in some cases. Especially, the helix and loop structures which are adopted by peptoids comprised mainly of α-chiral side chains, are well characterized. Recently, it is a significant issue to explore the relationships between peptoid structure and function.[34,35] Although it is not yet studied to connect peptoid structure with cell permeability, the side chains which were employed in this study, might mostly govern hydrophobic interactions to favor unstructured peptoids. Thus, the model conjugates were expected to provide a common phenomenon in the cell permeability of the cyclic and linear peptoids. For the attachment of the parent steroid to synthetic molecules for reporter gene-based assays, several dexamethasone derivatives were synthesized.[21−24,36] Among them, SDex-COOH was the best in regards to its binding affinity for the glucocorticoid receptor ligand-binding domain (GR LBD) and its compatibility with the solid-phase synthesis.

For the synthesis of cyclic peptoid−dexamethasone conjugates, Fmoc-Dpr(ivDde)-OH[37] was attached onto the Rink amide AM resin, where one amino group was used for the synthesis of the peptoid sequences and the other for the attachment of the dexamethasone derivative (Scheme 1). After every coupling step for the peptide units, the resin was treated with acetic anhydride to block any possible unreacted amino groups. ivDde is chemically quasi-orthogonal to Fmoc. Thus, Fmoc was removed with 50% morpholine first, instead of 20% piperidine in order to reduce the possible migration of ivDde to the α-amino group, and then Fmoc-*β*-Ala-OH was attached to the resins using PyBOP as a linker between dexamethasone moiety and peptoids.[37] For the peptoid cyclization in the later stage, Fmoc-Glu(*O*-2-PhiPr)-OH,[37] which contained two chemically orthogonal protecting groups (Fmoc and 2-PhiPr), was coupled with the resins. After the removal of Fmoc with 20% piperidine, we initially tried to make peptoids on the amino group of Glu using a well-known submonomer strategy, which employed acylation with bromoacetic acid (2M)/DIC[37] (3.2 M), followed by amination with primary amines (2 M) under microwave conditions.[26] However, the ivDde group was not removed when the resins were treated with 2% hydrazine,[23,37] meaning that the ivDde group must be altered. Thus, mass data of intermediates were checked after every synthetic step, and the byproducts whose masses were 40 more in the MALDI-TOF spectra than the expected products were identified. The amounts of the side products increased about 10−20% every coupling step for bromoacetic acid using DIC. The exact structures were unclear, but ivDde might have reacted with DIC. Next, we carried out the coupling process of bromoacetic acid under various reaction conditions in order to find the optimum conditions. Finally, the amount of DIC, reaction time and reaction temperature were found to be critical and the modified acylation conditions were identified using bromoacetic acid (1 M) and DIC (1.1 M) for 5 min at 35 °C without a microwave. After peptoid units were attached to the resins under these defined conditions, cyclic peptoids were synthesized on the resin through the selective cleavage of the acid-labile 2-PhiPr group with 1% TFA and the subsequent cyclization with PyBOP and HOBt, which were the best cyclization conditions for the development of cyclic peptoid libraries.[32,37] All of the cyclic peptoids from the 17-membered ring to 35-membered ring were efficiently cyclized, regardless of their ring size. The desired cyclic peptoid−dexamethasone conjugates (**C4**−**C10**) were formed through the deprotection of ivDde with 2% hydrazine and coupling with SDex-COOH. These conjugates were cleaved from the resins with 92% TFA containing 3% triisopropylsilane and 5% water, purified through reverse-phase HPLC

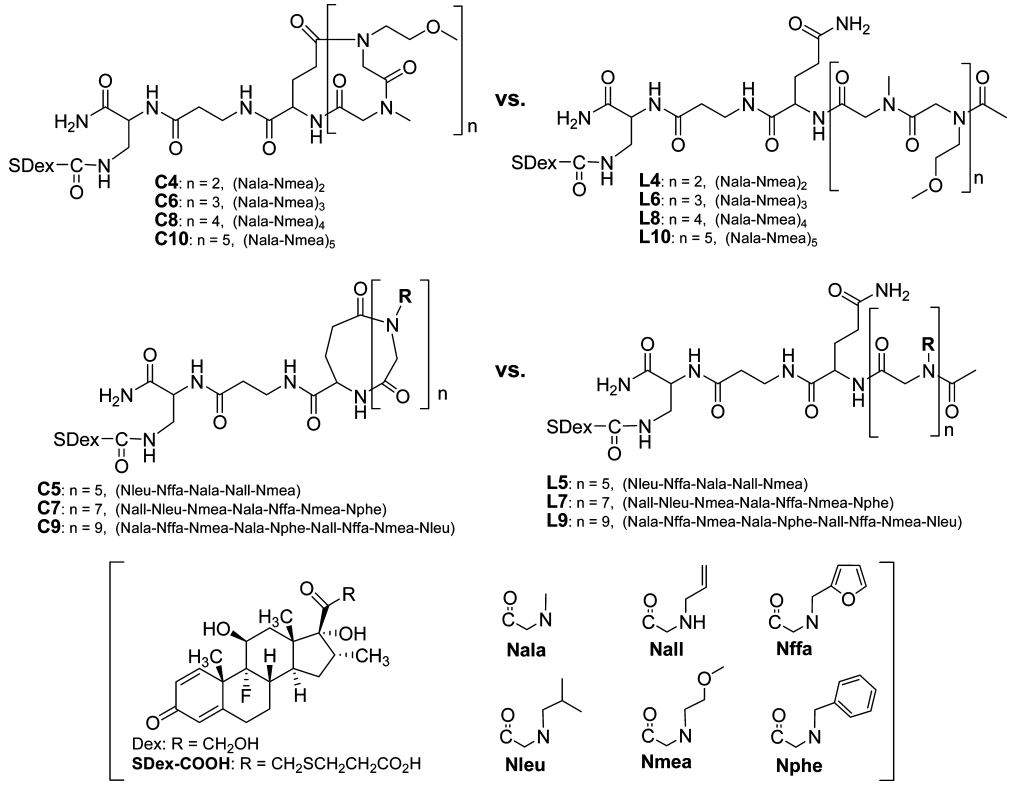
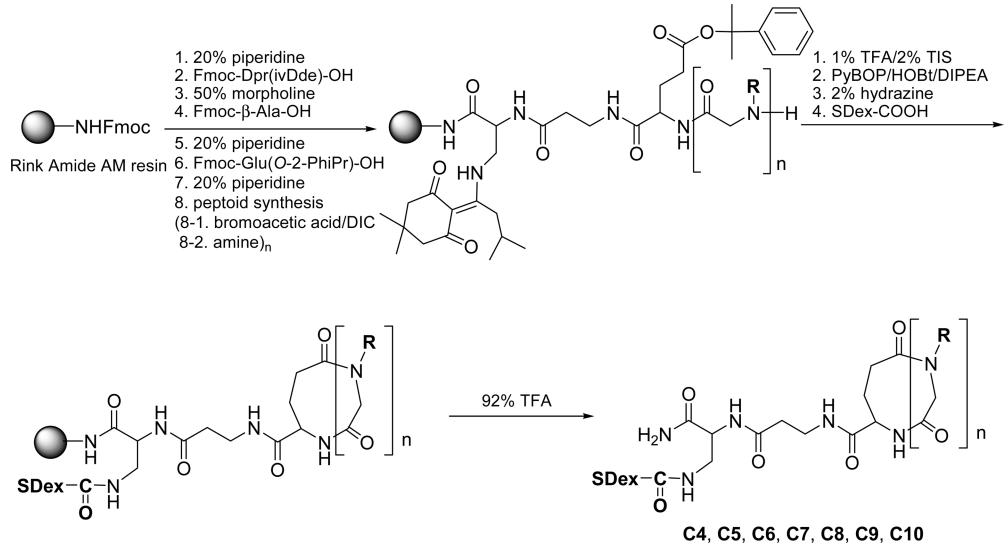


Figure 2. Chemical structures of the cyclic and linear peptoid−dexamethasone conjugates.

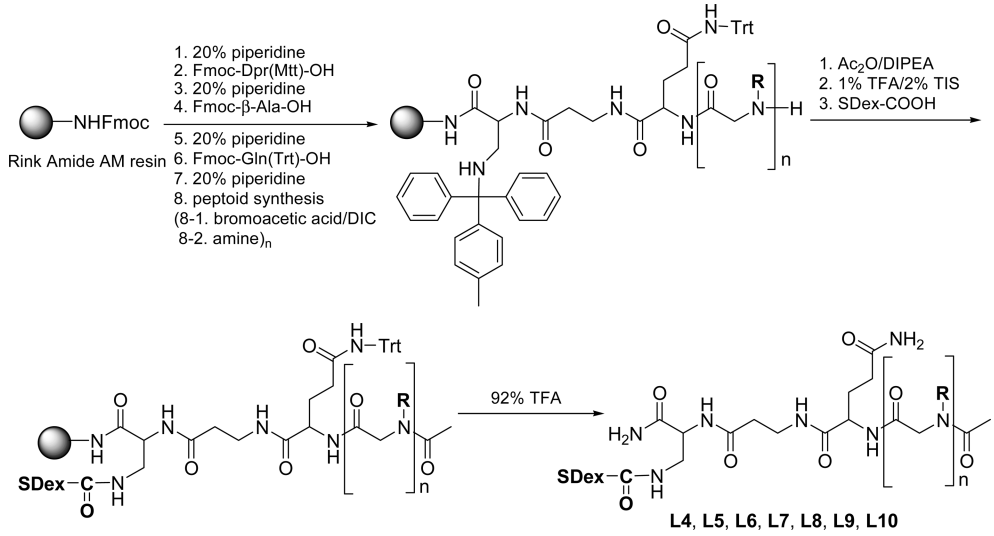
Scheme 1. Solid-Phase Synthesis of the Cyclic Peptoid−Dexamethasone Conjugates



and lyophilized in a freeze-dryer. The formation of the conjugates was also fully confirmed using MALDI-TOF analysis.

The synthesis of the linear peptoid−dexamethasone conjugates was also straightforward (Scheme 2). Both carboxylic acid and the amino termini of the linear counterparts were neutralized in order to prevent ionization in the aqueous solution, and thus, they might be more fairly compared to their corresponding cyclic counterparts. Fmoc-Gln(*O*-Trt)-OH[37] was chosen instead of Fmoc-Glu(*O*-2-PhiPr)-OH, and the terminal amino groups of the linear peptoids were also acetylated. At first, even though Fmoc-Dpr(ivDde)-OH could have been used, another diaminopropionic acid derivative, Fmoc-Dpr(Mtt)-OH[37] containing the Mtt group that could be selectively removed with 1% TFA, was used instead. Fmoc was removed with 20% piperidine, and then Fmoc-β-Ala-OH was attached to the resins using HATU and HOBt.[37] Next, Fmoc-Gln(*O*-Trt)-OH was similarly coupled with β-alanine. After Fmoc was removed, the peptoid sequences were synthesized on the resin using the submonomer strategy through the subsequent treatment of bromoacetic acid/DIC and amines under microwave.[26] The terminal amino group of the peptoids was thoroughly capped with acetic anhydride. In the final step, the Mtt group was selectively removed with 1% TFA, and then the free amino group was coupled with SDex-COOH. The desired linear peptoid−dexamethasone conjugates (**L4**−**L10**) were released from the

Scheme 2. Solid-Phase Synthesis of the Linear Peptoid−Dexamethasone Conjugates



resins with 92% TFA, purified through reverse-phase HPLC and lyophilized in a freeze-dryer.

Conclusion

A series of cyclic and linear peptoid−dexamethasone conjugates (**L4**−**L10** and **C4**−**C10**) were rationally designed and efficiently synthesized on solid-phase for systematic cell permeability studies using reporter gene-based assays. Cyclic peptides and their cyclic analogs have received a great deal of attention because of their numerous interesting biological activities and their challenging chemical synthesis. It has also been hypothesized that they might improve the cell permeability compared to linear molecules by providing the internal hydrogen bonding and generally decreasing the conformational flexibility. The model compounds in this study should be used to reveal how the cell permeability of cyclic molecules is affected by several physicochemical properties, especially, the reduced conformational flexibility and the ring size. Additionally, several chemically orthogonal protecting groups, such as Fmoc, ivDde, Mtt, and 2-PhiPr, were employed in this work. The efficient cyclization conditions were also determined, regardless of the ring sizes of the cyclic peptoids. Especially, the optimum conditions were identified for the attachment of peptoid units onto compounds containing the ivDde group. Thus, our synthetic strategy can provide a robust platform for postchemical modifications of various molecular scaffolds in solid-phase or solution-phase syntheses. The relative cell permeability studies of the model compounds using luciferase assays and in vitro GR binding assays will be reported elsewhere in due course.

Experimental Section

Materials and Equipment. All of the commercial reagents were used as obtained without further purification. The primary amines that were used in this study were methylamine, allylamine, isobutylamine, 2-methoxyethylamine, furfurylamine, and benzylamine. Rink amide AM resin (200−400 mesh, capacity: 0.62 mmol/g) and Rink amide AM resin LL (100−200 mesh, capacity: 0.35 mmol/g) were purchased from Novabiochem. Preparative HPLC was performed on a Shimadzu binary HPLC system with a C18 reverse-phase column using a gradient elution of water/acetonitrile with 0.1% TFA. MS (MALDI-TOF) was performed on a Voyager-DE STR biospectrometry workstation (Applied Biosystems) with α-hydroxy cinnamic acid as the matrix. The peptides were synthesized in an incubator shaker (JEIO TECH, model SI-600R), and the peptoids were synthesized in the incubator shaker without a microwave or in a microwave oven (Daewoo, model KR-B200R) at a power of 100 W. Standard glass peptide synthesis vessels (Chemglass) were used for the both syntheses in the incubator shaker and the microwave oven.

General Procedure for the Synthesis of Cylic Peptoid−Dexamethasone Conjugates. Rink amide AM resins LL (20 μmol) swelled in DMF at room temperature for 1 h. Then DMF was drained, and the beads were incubated with 20% piperidine for 30 min. The beads were thoroughly washed with DMF (8 × 3 mL) and then treated with Fmoc-Dpr(ivDde)-OH (2.5 equiv), HATU (2.5 equiv), HOBt (2.5 equiv), and DIPEA (10 equiv) in DMF for 2 h. The beads were thoroughly washed with DMF (8 × 3 mL) again and treated with acetic anhydride (10 equiv) and DIPEA (10 equiv) in DMF for 1 h to block any possible unreacted amino groups. The resins were treated with 50% morpholine in DMF twice for 15 min to reduce the possible migration of ivDde to the α-amine group during the Fmoc deprotection. After the resins were washed with DMF, they were suspended in DCM containing Fmoc-*β*-Ala-OH (5 equiv) and DIPEA (10 equiv). Then PyBOP (5 equiv) was added to the slurry at −40 °C, and the mixture was agitated for 30 min at −40 °C, and for 2 h at rt. The beads were thoroughly washed with DMF (8 × 3 mL) and again treated with acetic anhydride (10 equiv) and DIPEA (10 equiv) in DMF for 1 h. After the removal of Fmoc with 20% piperidine, the resins were coupled with Fmoc-Glu(*O*-2-PhiPr)-OH (2.5 equiv) in the presence of HATU (2.5 equiv), HOBt (2.5 equiv), and DIPEA (10 equiv) in DMF for 2 h. Any possible unreacted amino groups were also blocked with acetic anhydride. The peptoids were synthesized employing a modified submonomer strategy to make ivDde group safe.

After the removal of Fmoc with 20% piperidine, the beads were thoroughly washed with DMF (8 × 3 mL) and then treated with bromoacetic acid (1 M in DMF, 1 mL) and diisopropylcarbodiimide (DIC) (1.1 M in DMF, 1 mL) in an incubator for 5 min at 35 °C without a microwave. The beads were thoroughly washed with DMF (8 × 3 mL) and then treated with the primary amines (1−2 M in DMF, 1.5 mL) twice for 12 s in a microwave oven with a power of 100 W. Both acylation with bromoacetic acid and displacement of the bromide using the primary amines were successively repeated to form the desired peptoid sequences. The 2-PhiPr group was selectively deprotected with the treatment of 1% TFA and 2% triisopropylsilane in DCM twice for 30 min to synthesize the cyclic peptoids. After the resins were thoroughly washed with 5% DIPEA in DCM and then DCM, cyclization was carried out using PyBOP (3 equiv), HOBt (3 equiv), and DIPEA (10 equiv) in DMF twice for 8 h. The formation of the cyclic peptoids was confirmed by MALDI-MS after a tiny amount was cleaved from the resin. For the coupling of SDex-COOH,[23] ivDde was removed using 2% hydrazine and 50% allyl alcohol in DMF twice for 10 min. After the resins were thoroughly washed with DMF, SDex-COOH (2.5 equiv) was coupled with HATU (2.5 equiv), HOBt (2.5 equiv), and DIPEA (10 equiv) in DMF for 2 h. The desired products were released from the resins using 92% TFA containing 3% triisopropylsilane, and 5% water for 3 h, purified using reverse-phase HPLC using a gradient elution of water/acetonitrile with 0.1% TFA and lyophilized in a freeze-dryer. The cyclic peptoid−dexamethasone conjugates were confirmed using MALDI-TOF analysis. MS (MALDI-TOF): *m/z* **C4** calcd for $C_{52}H_{78}FN_9NaO_{15}S$ 1142.5, found 1142.6 $[M + Na]^+$; **C5** calcd for $C_{62}H_{89}FN_{10}NaO_{16}S$ 1303.6; found 1303.8 $[M + Na]^+$; **C6** calcd for $C_{60}H_{92}FN_{11}NaO_{18}S$ 1328.6, found 1328.4 $[M + Na]^+$; **C7** calcd for $C_{76}H_{107}FN_{12}NaO_{19}S$ 1565.7, found 1565.9 $[M + Na]^+$; **C8** calcd for $C_{68}H_{106}FN_{13}NaO_{21}S$ 1514.7, found 1514.8 $[M + Na]^+$; **C9** calcd for $C_{86}H_{119}FN_{14}NaO_{22}S$ 1773.8, found 1773.5 $[M + Na]^+$; **C10** calcd for $C_{76}H_{120}FN_{15}NaO_{24}S$ 1700.8, found 1700.5 $[M + Na]^+$.

General Procedure for the Synthesis of Linear Peptoid−Dexamethasone Conjugates. Rink amide AM resins (20 μmol) swelled in DMF at room temperature for 1 h. DMF was drained, and the beads were incubated with 20% piperidine for 30 min. The beads were thoroughly washed with DMF (8 × 3 mL) and then treated with Fmoc-Dpr(Mtt)-OH (2.5 equiv), HATU (2.5 equiv), HOBt (2.5 equiv), and DIPEA (10 equiv) in DMF for 2 h. After being thoroughly washed with DMF (8 × 3 mL) again, the beads were treated with acetic anhydride (10 equiv) and DIPEA (10 equiv) in DMF for 1 h to block any possible unreacted amino groups. After the Fmoc group was cleaved with 20% piperidine, the resins were washed with DMF (8 × 3 mL) and then treated with Fmoc-β-Ala-OH (5 equiv), HATU (5 equiv), HOBt (5 equiv), and DIPEA (10 equiv) in DMF for 2 h. The beads were thoroughly washed with DMF (8 × 3 mL) and again treated with acetic anhydride (10 equiv) and DIPEA (10 equiv) in DMF for 1 h. After the removal of Fmoc with 20% piperidine, the resins were coupled with Fmoc-Gln(Trt)-OH (2.5 equiv) in the presence of HATU (2.5 equiv), HOBt (2.5 equiv), and DIPEA (10 equiv) in DMF for 2 h. Any possible unreacted amino groups were also blocked with acetic anhydride. The peptoids was synthesized using a conventional submonomer strategy under microwave conditions. After the removal of Fmoc with 20% piperidine, the beads were thoroughly washed with DMF (8 × 3 mL) and then treated with bromoacetic acid (2 M in DMF, 1 mL) and diisopropylcarbodiimide (DIC) (3.2 M in DMF, 1 mL) twice for 12 s in a microwave oven that was set to a power of 100 W. The beads were thoroughly washed with DMF (8 × 3 mL) and then treated with the primary amines (1−2 M in DMF, 1.5 mL) twice for 12 s in a microwave oven with a power of 100 W. Both the acylation with bromoacetic acid and displacement of the bromide using the primary amines were successively repeated to form the desired peptoid sequences. The terminal amino groups of the peptoids were thoroughly capped with acetic anhydride (10 equiv) in the presence of DIPEA (10 equiv) in DMF for 2 h. For the coupling of SDex-COOH,[23] the Mtt group was selectively deprotected through the treatment of 1% TFA and 2% triisopropylsilane in DCM twice for 30 min. The resins were thoroughly washed with 5% DIPEA in DCM and then DCM, and SDex-COOH (2.5 equiv) was coupled using HATU (2.5 equiv), HOBt (2.5 equiv), and DIPEA (10 equiv) in DMF for 2 h. The desired products were released from the resins using 92% TFA containing 3% triisopropylsilane and 5% water for 3 h, purified using reverse-phase HPLC using a gradient elution of water/acetonitrile with 0.1% TFA and lyophilized in a freeze-dryer. The formation of the linear peptoid-dexamethasone conjugates was confirmed using MALDI-TOF analysis. MS (MALDI-TOF): *m/z* **L4** calcd for $C_{54}H_{83}FN_{10}NaO_{16}S$ 1201.6, found 1201.4 $[M + Na]^+$; **L5** calcd for $C_{64}H_{94}FN_{11}NaO_{17}S$ 1362.6, found 1362.6 $[M + Na]^+$; **L6** calcd for $C_{62}H_{97}FN_{12}NaO_{19}S$ 1387.7, found 1388.2 $[M + Na]^+$; **L7** calcd for $C_{78}H_{112}FN_{13}NaO_{20}S$ 1624.8, found 1624.6 $[M + Na]^+$; **L8** calcd for $C_{70}H_{111}FN_{14}NaO_{22}S$ 1573.8, found 1573.7 $[M + Na]^+$; **L9** calcd for $C_{88}H_{124}FN_{15}NaO_{23}S$ 1832.9, found 1833.3 $[M + Na]^+$; **L10** calcd for $C_{78}H_{125}FN_{16}NaO_{25}S$ 1759.9, found 1759.9 $[M + Na]^+$.

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Supporting Information Available. Useful experimental procedures, MALDI-TOF spectra of target compounds and ^{1}H NMR spectra of representative compounds (**C4/L4** and **C7/L7**). This material is available free of charge via the Internet at http://pubs.acs.org.

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(37) Abbreviation: Fmoc, 9-fluorenylmethyloxycarbonyl; Dpr, diaminopropionic acid; ivDde, 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl; PyBOP, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluoro-phosphonate; 2-PhiPr, 2-phenylisopropyl; DIC, *N, N′*-diisopropylcarbodiimide; SDexCOOH, dexamethasone-21-thiopropionic acid; TFA, trifluoroacetic acid; HOBt, 1-hydroxybenzotriazole; Trt, trityl; Mtt, 4-methyltrityl; HATU, 2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate.

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