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Quantitative Comparison of the Relative Cell Permeability of Cyclic and Linear Peptides

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SUMMARY

Cyclic peptides are of considerable interest as potential protein ligands. It has been postulated that cyclic molecules might be more cell permeable than their linear counterparts due to their reduced conformational flexibility. We report a study that tests this hypothesis by using a quantitative, reporter gene-based assay that measures the relative cell permeability of steroid conjugates of molecules of interest. We demonstrate that cyclic peptides are, in fact, not generally more permeable than their linear counterparts.

INTRODUCTION

Cyclic peptides and depsipeptides have many interesting biological activities [1–4]. They are usually more resistant to proteolytic degradation than their linear isomers [5, 6]. In some cases, cyclic peptides may bind more tightly to their protein targets due to their more restricted conformational flexibility [7, 8]. It has also been suggested that this same reduction in conformational flexibility of cyclic peptides should improve the cell permeability of these molecules [6, 9–12]. However, few careful experimental tests of the latter hypothesis have been published. Herein, we report the synthesis of cyclic and linear peptide scaffolds decorated with various side chains and test their relative cell permeability by using a quantitative assay developed recently in our laboratory [13]. We find that, at least for the family of compounds explored here, cyclic molecules do not exhibit significantly higher cell permeability than their linear analogs.

The assay that we employed (shown schematically in Figure S1 in the Supplemental Data available with this article online) is a slight variation on a method reported previously [13]. Briefly, a steroid conjugate of the molecule of interest is incubated with cells that express high levels of an artificial transactivator protein comprised of the Gal4 DNA-binding domain, the glucocorticoid receptor ligand-binding domain, and the VP16 transactivation domain (Gal4 DBD-GR LBD-VP16). The cells also contain a Gal4-responsive firefly luciferase reporter gene (pG5B) and a second reporter plasmid encoding *Renilla reniformis* luciferase (pRL-SV40), which serves as a transfection control. Gal4 DBD-GR LBD-VP16 is a potentially potent transcriptional activator, but, in the absence of a steroid ligand, it is trapped in an inactive form by the high-affinity interaction of Hsp90 with the GR LBD. If the steroid-containing chimera is cell permeable, then its entry into the cell will result in release of Gal4 DBD-GR LBD-VP16 from Hsp90 in a dose-dependent fashion and subsequent induction of firefly luciferase reporter gene expression. Note that there is no evidence for facilitated diffusion of this class of steroids; thus, the assay measures the extent to which the molecule fused to the steroid impedes (or not) its passive diffusion across the membrane. By comparing the IC_{50} values observed for different molecules conjugated to the same steroid, their relative cell permeabilities can be compared quantitatively after correcting for possible differences in their affinity for the GR LBD. This is measured for each molecule in vitro by using a competitive fluorescence polarization assay, as described [13].

RESULTS AND DISCUSSION

Synthesis of Cyclic and Linear Peptides

For this study, we created the compounds shown in Figure 1. Both termini of the linear peptides were capped to prevent their ionization in aqueous solution and thus allow them to be compared more fairly to their cyclic counterparts. First, we made cyclic (cyclo-[Glu(Fmoc)-Dpr(Alloc)-Dpr(ivDde)-Dpr]-) and linear (Gln[Fmoc][Trt]-Dpr[Alloc]-Dpr[ivDde]-Dpr[Ac]-) scaffolds on Rink amide resin (Alloc, allyloxycarbonyl; ivDde, 1-[4,4-dimethyl-2,6-dioxocyclohex-1-ylidene]-3-methylbutyl; Dpr, diaminopropionic acid). These scaffolds have three chemically orthogonal protecting groups: Fmoc, Alloc, and ivDde (Figure 2). This allows stepwise deprotection and substitution at each step. The scaffold approach is useful in that it avoids the problem of variable cyclization yields for different linear precursors. A cyclic scaffold was made on resin by selective cleavage of acid-labile protecting groups (4-methyltrityl [Mtt] and 2-phenylisopropyl [2-PhiPr]) with 1% TFA and subsequent cyclization with benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluoro-phosphonate (PyBOP) and 7-aza-1-hydroxybenzotriazole (HOAt) [14, 15]. Side chain manipulation of cyclic and linear scaffolds was carried out sequentially by the initial deprotection of the piperidine-labile Fmoc and coupling with acids, followed by the deprotection of the hydrazine-labile ivDde [16] and coupling with acids, and, finally, the deprotection of the Pd-labile Alloc

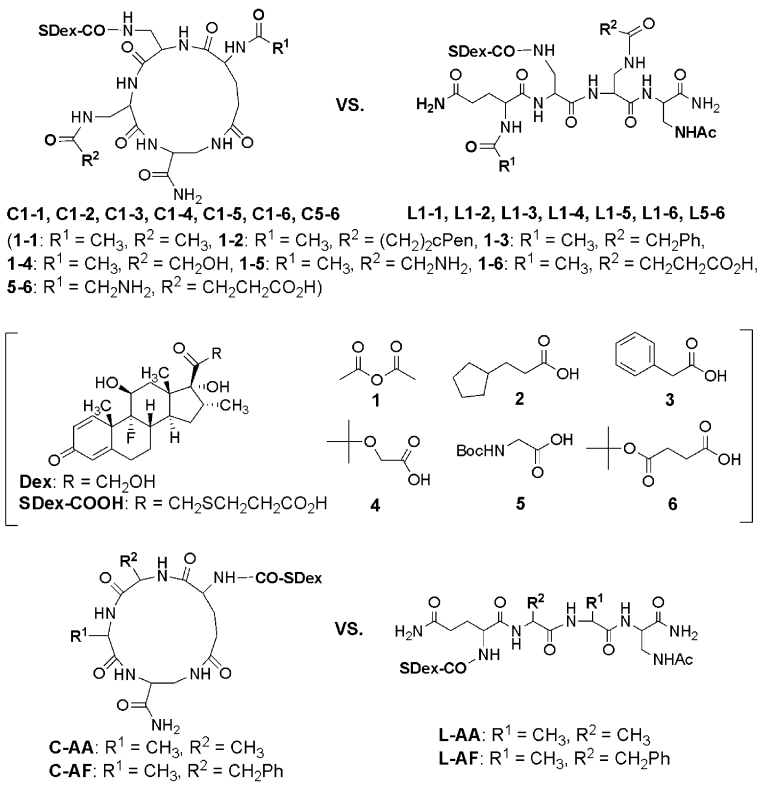

Figure 1. Chemical Structures of Cyclic and Linear Peptides

[17], followed by coupling with the dexamethasone derivative. The steroid that we employed in this study (SDex-COOH) is different from those employed in our previous experiments in which this assay was used [13, 18]. SDex-COOH has a very high affinity for the GR LBD, making it useful for the analysis of sparingly cell-permeable compounds such as peptides (vide infra), and is compatible with solid-phase synthesis [19]. The desired SDex-COOH conjugates were purified by reverse-phase HPLC after sequential manipulation as explained above (Figure 2).

Measurement of the In Vitro GR-Binding Affinities

First, we measured the in vitro GR-binding affinity of each conjugate by using a fluorescence polarization-based assay that monitors displacement of a fluorescein-labeled dexamethasone derivative from purified GR by the synthetic conjugate. The IC_{50} values of each compound derived from this analysis (Figure 3) were all in the high nanomolar range (Table 1). The cyclic and the corresponding linear peptides had similar GR-binding affinities, and the GR-binding patterns were also quite similar in both series.

Luciferase Assays

Next, we carried out luciferase assays with HeLa cells that had been transfected with a plasmid directing the expression of Gal4 DBD-GR LBD-VP16 as well as the constitutive and Gal4-responsive luciferase reporter plasmids, as described above. Luminescent intensities were measured after incubating the cells for 40 hr with the compound of interest (Figure 4). Gal4-responsive firefly luciferase activity was normalized to the constitutive *Renilla reniformis* luciferase activity and then to the background signal to obtain the EC_{50} values and maximum levels of induction from the titration curves (see Table 1). The relative cell permeabilities were then gleaned from these data after correcting for differences in GR LBD affinity, which were small.

Cyclic Peptides Are Not More Cell Permeable than Their Linear Counterparts

In both the cyclic and linear peptide series, hydrophobic- and aromatic-functionalized compounds (**C/L1-2** and **C/L1-3**) have lower EC_{50} values and higher maximum fold induction, indicating that they are more cell permeable than the polar or charged compounds, as might have been expected.

When the permeability measurements for the corresponding cyclic and linear peptides were compared, the simple cyclic peptide, **C1-1**, had an EC_{50} value of 41.8 μM, while the corresponding linear peptide, **L1-1**, had an EC_{50} value of 8.1 μM, showing that the linear isomer is about 5.7 times more cell permeable than its cyclic counterpart. This trend was also observed for the other isomeric pairs; the linear molecule exhibited an apparent permeability that was 2.0–6.2 times greater than that of its cyclic analog regardless of the side chain moieties (see Table 1). Obviously, these results are inconsistent with the idea that cyclic peptides are generally more permeable than their linear counterparts due to reduced freedom of conformation [6, 10]. It is important to point out that, under the conditions of the assay, both the linear and cyclic peptides

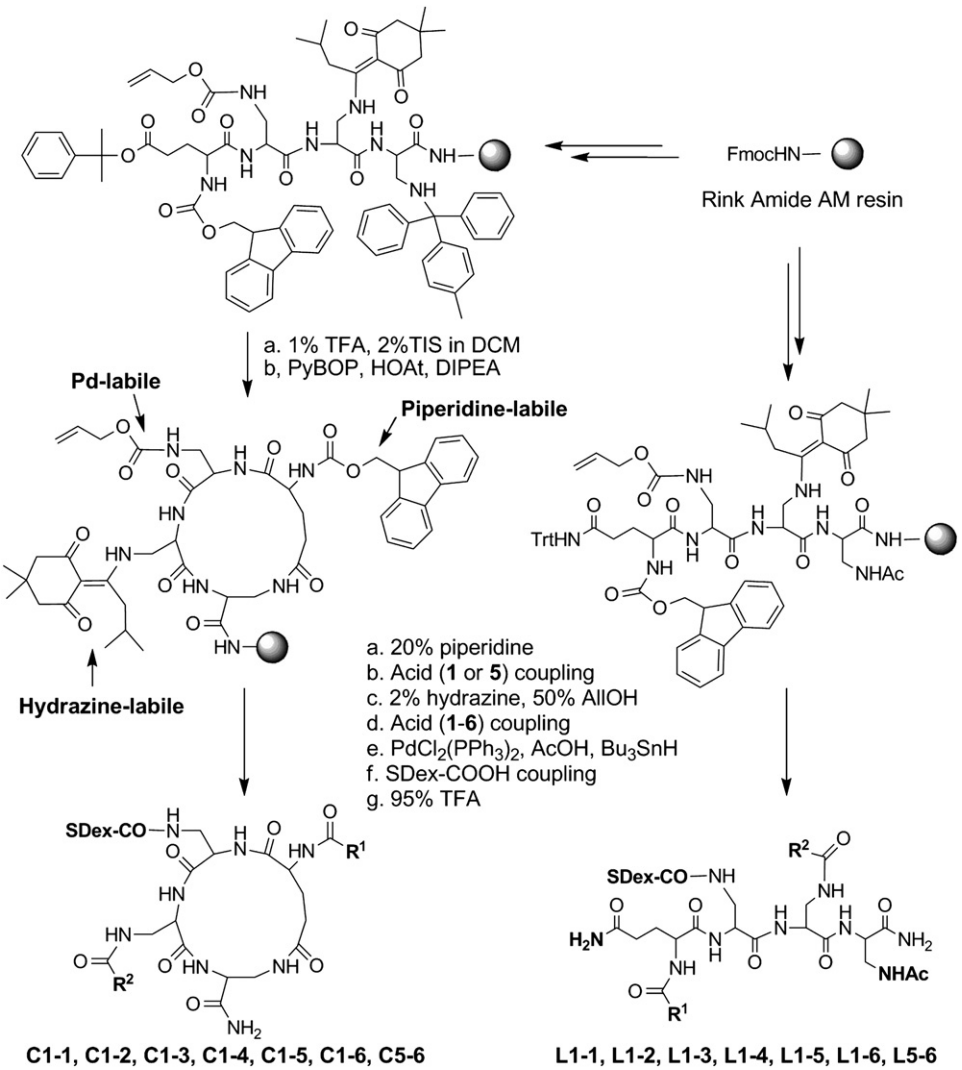

Figure 2. Synthesis of Cyclic and Linear Peptides

were quite stable. Mass spectrometry was employed to monitor the level of each peptide relative to a proteolytically insensitive internal standard, and the data showed no more than a 5% loss of the parent compounds over the course of the assay, and most or all of this could be explained by the noise in the measurement (data not shown). There was no significant difference between the stability of the linear and cyclic peptides.

We also synthesized SDex-COOH derivatives (**C/L-AA** and **C/L-AF**) of peptides lacking amide functionality in their side chains as analogs of **C/L1-1** and **C/L1-3**. Generally, internal and intramolecular hydrogen bond potential is thought to be critical for passive membrane permeability [7–10], and it may be that the propensity of side chain amides and main chain amides to form intramolecular hydrogen bonds might be different in linear and cyclic molecules, thus justifying a comparison. The data (Figures S4 and S7) reveal that, again, there is little difference in the relative cell permeabilities of the analogous linear and cyclic molecules (**C/L-AA** and **C/L-AF**), further buttressing the conclusion reached.

While not the focus of this study, it is interesting to note that **L1-1** and **L1-3** had EC_{50} values of 8.1 μM and 0.84 μM, respectively, while the related compounds **L-AA** and **L-AF** had EC_{50} values of 36.5 μM and 62.2 μM, respectively. These data indicate that **L1-1** and **L1-3** are much more cell permeable than their analogs, **L-AA** and **L-AF**. These data may suggest that side chain amides of linear peptides are able to form intramolecular hydrogen bonds that facilitate cell permeability. However, more directed studies of compounds designed to test this specific hypothesis would be required in order to make general statements of this type.

Validation of the Luciferase Assay System by a Fluorescence Microscopy Assay

To further validate our luciferase assay system, we took advantage of conventional fluorescence microscopy

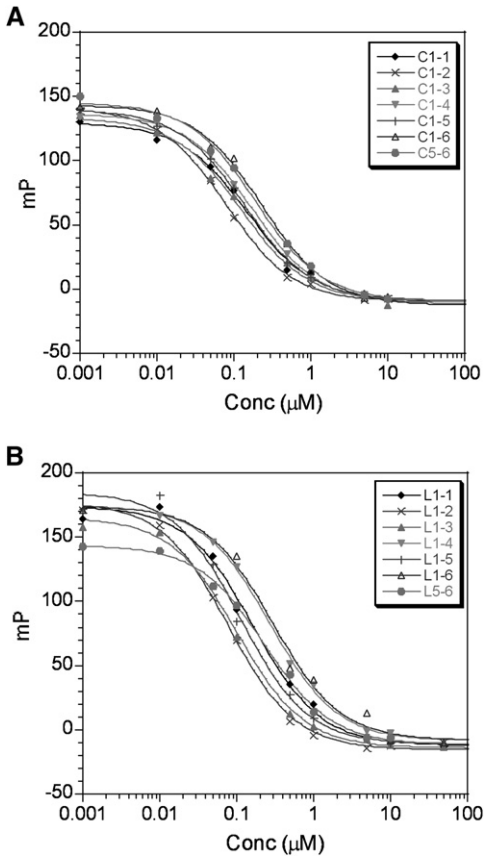

Figure 3. Fluorescence Polarization Data for Measurement of In Vitro GR-Binding Affinity

(A) Data for cyclic peptides.

(B) Data for linear peptides.

The displacement of a fluorescein-labeled dexamethasone was monitored by a decrease in fluorescence polarization. The data were plotted as the millipolarization units (mP) versus the concentration of the competitors. Derived IC_{50} values are shown in Table 1.

[13]. We chose to compare two pairs with a relatively high (**C/L1-3**) and low (**C/L1-4**) permeability difference, respectively. Two fluorescein-labeled cyclic and linear peptide pairs (**C/L1-3-FL** and **C/L1-4-FL**) were synthesized by the attachment of an acetyl-ethyleneglycol-ethylamine (AEEA) linker and then carboxyfluorescein instead of SDex-COOH (Figure S8). HeLa cells were incubated with 20 μM of the fluorescein-labeled compound or carboxyfluorescein as a control for 4 hr. The fluorescence images were taken after the cells were washed with 1× PBS, fixed with 4% formaldehyde, and mounted with VECTASHIELD medium containing 4′,6-diamidino-2-phenylindole (DAPI). The results showed that all compounds are quite cell permeable (Figure S9). While the fluorescence microscopy assays are poorly quantitative and the cell permeability differences encountered with our assay system are also relatively small, this similarity in the brightness of the images of cells treated with fluorescein-labeled cyclic and linear peptides is consistent with the data obtained by using our method.

Evaluation of Physicochemical Properties

Several physicochemical properties, including molecular size/volume, hydrophobicity, hydrogen bond potential, polar surface area, desolvation energy, solubility, etc. have been considered as contributors to cell permeability [20–23]. Among these, particular importance has been placed on the desolvation of hydrated hydrogen bonds in explaining the differences in the permeability between linear and cyclic peptides. This is because the gross physical properties of cyclic and linear peptides of the same sequence are almost identical and thus are unlikely to explain any large degree of difference in their permeabilities. However, the presumably different conformational preferences of these molecules could easily lead to a large difference in internal hydrogen bonding and thus the amount of desolvation required to pass from the aqueous phase into a lipid membrane. For example, Rezai et al. investigated the relationship between the conformation and membrane permeability of cyclic peptides by using a parallel artificial membrane permeability assay (PAMPA) [24], an NMR study, and computational modeling [10]. They were able to develop a model in which conformation-dependent intramolecular hydrogen bonding could explain the different apparent permeabilities of various cyclic peptides [9]. They suggested that a similar argument could be made to rationalize a single example they presented of a cyclic peptide that was much more permeable than its linear counterpart (in the PAMPA assay). It seems clear that more intramolecular hydrogen bonding would indeed increase cell permeability. However, in addressing the major point of this paper, the real question is whether cyclic peptides will generally have a greater amount of intramolecular hydrogen bonding than linear peptides. From the results presented here, it would seem that the answer is no. We emphasize that detailed conformational studies have not been done on the peptides employed in this study, so any discussion of the degree of intramolecular hydrogen bonding would be quite speculative. Nonetheless, the results are quite clear and show that the cyclic peptides studied here are not more permeable than their linear counterparts. Since the linear and cyclic molecules used here have very similar partition coefficients, total polar surface areas, etc., and have similar cell permeabilities, one is forced to conclude that they do not differ drastically in the degree of intramolecular hydrogen bonding. Therefore, the hypothesis that cyclic peptides will, in general, be easier to desolvate than linear peptides does not seem to be true.

SIGNIFICANCE

We assessed quantitatively the relative cell permeability of comparable steroid conjugates of cyclic and linear peptides by using a reporter gene-based assay in HeLa cells. We find no evidence that indicates that the cyclic molecules are generally more cell permeable. Indeed, the opposite appears to be true, although in no case is the difference very large. Cyclic peptides

Table 1. Summary of Parameters from In Vitro GR-Binding Affinity and Luciferase Assays

Compound[a]	EC_{50}[b] (μM)	IND_{max}[b] (Fold)	IC_{50}[c] (μM)	Relative Permeability Factor[d]
C1-1	41.8 ± 6.3	2711 ± 246	148 ± 22	
C1-2	6.1 ± 0.8	3020 ± 128	79 ± 4	
C1-3	6.3 ± 1.1	2690 ± 114	124 ± 16	
C1-4	23.5 ± 6.6	2724 ± 358	173 ± 8	
C1-5	24.4 ± 7.5	1643 ± 241	137 ± 9	
C1-6	13.0 ± 2.0	2054 ± 120	230 ± 26	
C5-6	10.2 ± 2.3	433 ± 36	193 ± 24	
L1-1	8.1 ± 2.8	1463 ± 142	164 ± 30	5.7
L1-2	3.0 ± 0.4	3722 ± 136	79 ± 6	2.0
L1-3	0.84 ± 0.1	3125 ± 82	102 ± 10	6.2
L1-4	16.5 ± 5.5	1849 ± 209	270 ± 26	2.2
L1-5	4.7 ± 1.7	1731 ± 169	112 ± 21	4.2
L1-6	3.8 ± 0.5	1518 ± 56	295 ± 68	4.4
L5-6	3.2 ± 1.3	1401 ± 137	233 ± 26	3.8
C-AA	21.2 ± 6.6	1497 ± 109	141 ±14	
C-AF	94.2 ± 37	1781 ±288	38 ± 4	
L-AA	36.5 ± 7.8	2240 ±135	97 ± 11	0.40
L-AF	62.2 ± 18	1242 ± 127	67 ±10	2.7

[a] As references, **SDex-CONH$_2$**: EC_{50} = 2.8 μM, IC_{50} = 36.3 nM; isobutyl amide of SDex-COOH (**SDex-CO-IBA**): EC_{50} = 567 nM, IC_{50} = 14.1 nM (see Figures S3 and S6).
[b] Data from luciferase assays.
[c] Data from in vitro GR-binding assays.
[d] The relative permeability of linear peptides compared to the corresponding cyclic peptides, when combined with in vitro GR-binding affinity; for example, (EC_{50}[**C1-1**]/EC_{50}[**L1-1**])/(IC_{50}[**C1-1**]/IC_{50}[**L1-1**]).

remain a potentially intriguing class of protein ligands due to their enhanced proteolytic stability and the hope that they might exhibit tighter binding to proteins than linear peptides; however, they cannot be relied upon to exhibit greater cell permeability.

EXPERIMENTAL PROCEDURES

Synthesis of the Cyclic Peptide Scaffold on the Resin

The synthesis of the cyclic peptide scaffold was performed by employing conventional peptide coupling chemistry. Rink amide AM resins in DMF were allowed to swell at room temperature for 1 hr. After DMF was drained, 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 eq.), HBTU (1.95 eq.), HOBt (1.95 eq.), and DIPEA (10 eq.) in DMF for 1.5 hr. After deprotection of Fmoc group with 20% piperidine, the beads were similarly coupled with Fmoc-Dpr(ivDde)-OH (2 eq.). In order to reduce possible migration of ivDde to the α-amine group during Fmoc deprotection, the resins were treated twice, for 15 min each, with 50% morpholine in DMF. After being washed with DMF, the resins were suspended in DCM containing Fmoc-Dpr(Alloc)-OH (2 eq.) and DIPEA (10 eq.). After PyBOP (2 eq.) was added to the slurry at −40°C, it was agitated for 30 min at −40°C, then for 2 hr at room temperature. After deprotection of Fmoc with 20% piperidine, the resins were coupled with Fmoc-Glu(*O*-2-PhiPr)-OH (2 eq.). Both Mtt and 2-PhiPr groups were deprotected with 1% TFA and 2% triisopropylsilane in DCM. After the resins were thoroughly washed with both 5% DIPEA in DCM and DCM alone they were dried in vacuo for 2 hr. Cyclization was carried out with PyBOP (3 eq.), HOAt (3eq.), and DIPEA (10 eq.) in DMF and was performed twice (for 24 hr each time). The cyclic scaffold was confirmed by MALDI-MS [899 $(M + H)^+$] after cleavage from the resin.

Synthesis of the Linear Peptide Scaffold on the Resin

The synthesis of the linear peptide scaffold was similarly performed by employing conventional peptide coupling chemistry. Rink amide AM resins in DMF were allowed to swell at room temperature for 1 hr. After DMF was drained, 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 eq.), HBTU (1.95 eq.), HOBt (1.95 eq.), and DIPEA (10 eq.) in DMF for 1.5 hr. The Mtt group was removed with 1% TFA and 2% triisopropylsilane in DCM. After the resins were thoroughly washed with both 5% DIPEA in DCM and DCM alone, they were treated with acetic anhydride (20 eq.) and DIPEA (20 eq.) for 2 hr. After deprotection of the Fmoc group with 20% piperidine, the beads were similarly coupled with Fmoc-Dpr(ivDde)-OH (2 eq.). In order to reduce possible migration of ivDde to the α-amine group during Fmoc deprotection, the resins were treated twice, for 15 min each, with 50% morpholine in DMF. After being washed with DMF, the resins were suspended in DCM containing Fmoc-Dpr(Alloc)-OH (2 eq.) and DIPEA (10 eq.). After PyBOP (2 eq.) was added to the slurry at −40°C, it was agitated for 30 min at −40°C, then for 2 hr at room temperature. After deprotection of Fmoc with 20% piperidine, the resins were coupled with Fmoc-Gln(Trt)-OH (2 eq.). The linear scaffold was confirmed by MALDI-MS [958 $(M + H)^+$] after cleavage from the resin.

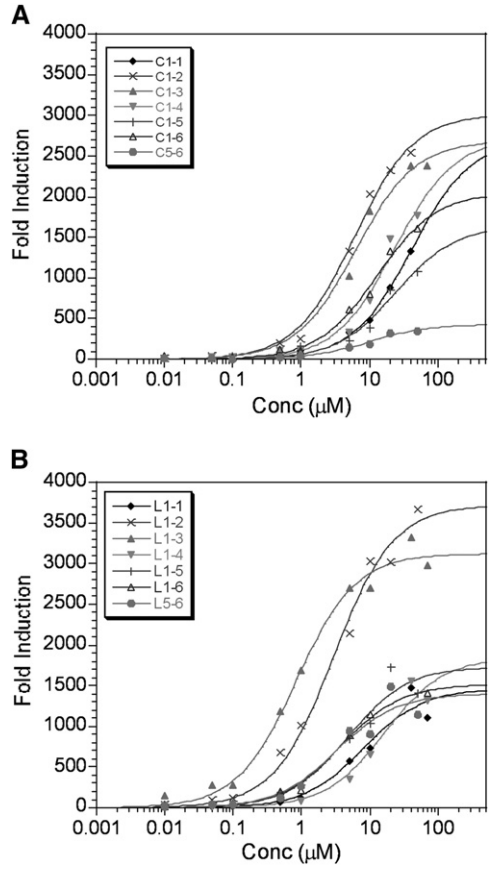

Figure 4. Dose-Dependent Induction of Luciferase Expression

(A) Data for cyclic peptides.

(B) Data for linear peptides.

Luminescent intensities were measured 40 hr after treatment of each compound at various concentrations with a dual-luciferase reporter assay system. The data were plotted as the fold induction of luciferase activities versus the concentration of the compounds. EC_{50} values derived from these data are summarized in Table 1.

General Procedure for the Synthesis of SDex-CO-C/L Peptide Conjugates

SDex-CO-C/L peptide conjugates were synthesized by sequential deprotection of Fmoc, ivDde, and Alloc groups and conventional peptide coupling after each deprotection step by using cyclic or linear scaffolds on the resin. Fmoc was removed by using 20% piperidine in DMF for 30 min, and ivDde was removed by using 2% hydrazine and 50% allyl alcohol in DMF (this was performed twice for 10 min each time). Alloc was deprotected by $PdCl_2(PPh_3)_2$ (0.1 eq.), acetic acid (3.5 eq.), and tributyltin hydride (3 eq.) in DCM (this was performed twice for 30 min each time), and the resins were then thoroughly washed with DCM (3 × 3 ml), 10% TEA in DCM (3 × 3 ml), DCM (3 × 3 ml), CH_3OH (3 × 3 ml), and DCM (3 × 3 ml). After each deprotection, each acid (5 eq.) was coupled with HATU (5 eq.), HOAt (5 eq.), DIPEA (10 eq.), and 2,6-lutidine (7 eq.) in DMF for 1.5 hr, and the acetyl group was introduced by using acetic anhydride (20 eq.) and DIPEA (20 eq.) in DMF for 2 hr. At the final step, SDex-COOH (2.5 eq.) was coupled with HATU (2.5 eq.), HOAt (2.5 eq.), DIPEA (10 eq.), and 2,6-lutidine (7 eq.) in DMF for 1.5 hr. The desired products were released from the resins by using 95% TFA containing 2.5% triisopropylsilane and 2.5% water and were purified by reverse-phase HPLC. The structures of **SDex-CO-C/L** peptide conjugates were confirmed by MALDI-TOF analysis. MS (MALDI-TOF): *m/z*: **C1-1**: calcd for $C_{43}H_{61}FN_8NaO_{12}S$ 955.4, found 955.4 $[M + Na]^+$; **C1-2**: calcd for $C_{49}H_{71}FN_8NaO_{12}S$ 1037.5, found 1037.4 $[M + Na]^+$; **C1-3**: calcd for $C_{49}H_{65}FN_8NaO_{12}S$ 1031.4, found 1031.4 $[M + Na]^+$; **C1-4**: calcd for $C_{43}H_{61}FN_8NaO_{13}S$ 971.4, found 971.5 $[M + Na]^+$; **C1-5**: calcd for $C_{43}H_{62}FN_9NaO_{12}S$ 970.4, found 970.4 $[M + Na]^+$; **C1-6**: calcd for $C_{45}H_{63}FN_8NaO_{14}S$ 1013.4, found 1013.5 $[M + Na]^+$; **C5-6**: calcd for $C_{45}H_{64}FN_9NaO_{14}S$ 1028.4, found 1028.6 $[M + Na]^+$; **L1-1**: calcd for $C_{45}H_{66}FN_9NaO_{13}S$ 1014.4, found 1014.2 $[M + Na]^+$; **L1-2**: calcd for $C_{51}H_{76}FN_9NaO_{13}S$ 1096.5, found 1096.5 $[M + Na]^+$; **L1-3**: calcd for $C_{51}H_{70}FN_9NaO_{13}S$ 1090.5, found 1090.2 $[M + Na]^+$; **L1-4**: calcd for $C_{45}H_{66}FN_9NaO_{14}S$ 1030.4, found 1030.3 $[M + Na]^+$; **L1-5**: calcd for $C_{45}H_{67}FN_{10}NaO_{13}S$ 1029.4, found 1029.4 $[M + Na]^+$; **L1-6**: calcd for $C_{47}H_{68}FN_9NaO_{15}S$ 1072.4, found 1072.5 $[M + Na]^+$; **L5-6**: calcd for $C_{47}H_{69}FN_{10}NaO_{15}S$ 1087.5, found 1087.3 $[M + Na]^+$.

Synthesis of C-AA, C-AF, L-AA, and L-AF

Compounds **C-AA**, **C-AF**, **L-AA**, and **L-AF** were synthesized by using Fmoc-Ala-OH or Fmoc-Phe-OH instead of Fmoc-Dpr(ivDde)-OH or Fmoc-Dpr(Alloc)-OH under conditions similar to those employed for the synthesis of cyclic or linear peptide scaffolds. The final products were also cleaved from the resins by using 95% TFA containing 2.5% triisopropylsilane and 2.5% water and were purified by reverse-phase HPLC. Their structures were confirmed by MALDI-TOF analysis. MS (MALDI-TOF): *m/z*: **C-AA**: calcd for $C_{39}H_{55}FN_6NaO_{10}S$ 841.4, found 841.5 $[M + Na]^+$; **C-AF**: calcd for $C_{45}H_{59}FN_6NaO_{10}S$ 917.4, found 917.5 $[M + Na]^+$; **L-AA**: calcd for $C_{41}H_{60}FN_7NaO_{11}S$ 900.4, found 900.4 $[M + Na]^+$; **L-AF**: calcd for $C_{47}H_{64}FN_7NaO_{11}S$ 976.4, found 976.5 $[M + Na]^+$.

Concentration Determination of SDex-COOH Conjugates

SDex-COOH conjugates were dissolved in DMSO. The concentration of each compound was determined by the distinct absorbance of dexamethasone at 242 nm with an extinction coefficient of 1.2×10^4 $M^{-1}cm^{-1}$.

In Vitro Competition GR-Binding Assays

The GR-binding affinity was determined by using the glucocorticoid receptor Competitor Assay Kit (Invitrogen). SDex-COOH conjugates were mixed with 1 nM fluormone and 4 nM recombinant human glucocorticoid receptor in 100 μl buffer and were incubated in the dark at 25°C for 1.5 hr. Fluorescence polarization was measured on a Panvera Beacon 2000 fluorometer (Invitrogen). The concentration of a SDex-COOH conjugate that results in a half-maximum decrease in the polarization value is defined as the IC_{50} of the compound, which is a measure of the relative binding affinity of the test compound for glucocorticoid receptor. The binding data were fit to the equation $(P = [1 - C/(IC_{50} + C)] \times [P_{max} - P_{min}] + P_{min})$ where C is the concentration of the test compound, P is the polarization of the sample, P_{max} is the polarization value observed when no competitor is added to the glucocorticoid receptor-fluormone complex, and P_{min} is the polarization value that represents maximum competition and was measured when 1 μM dexamethasone was used as the glucocorticoid receptor competitor.

Luciferase Assays

HeLa cells (~2.0×10^4 cells/well) were split in 96-well plates and maintained at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum. After 1 day, cells were transfected in OPTI-MEM I medium by the Lipofectamine Plus method (Invitrogen) with 0.101 μg total DNA, including 0.05 μg Gal4 DBD-GR LBD-VP16, 0.05 μg pG5B reporter plasmid, and 1 ng *Renilla reniformis* luciferase plasmid (pRL-SV40), which were prepared as previously mentioned [13]. After DNA transfection, cells were maintained at 37°C under 5% CO_2 for 3 hr. Cells in DMEM were treated with SDex-COOH conjugates dissolved in DMSO at various concentrations. Luciferase assays were conducted 40 hr after treatment of each compound with a dual-luciferase reporter assay system (Promega).

The luminescence was measured on a Sirius luminometer (Berthold detection systems). The value obtained was normalized with respect to the *Renilla reniformis* luciferase luminescence. The result for the control cells to which no ligand was added was treated as 1, the baseline for the induction. Other results were normalized based on this baseline and finally presented as the fold of inductions. The concentration of the SDex-COOH conjugate that results in a half-maximum increase of the induction level is defined as EC_{50}. The maximum induction level is defined as IND_{max}. The induction data were fit to the equation $(I = [C/(EC_{50} + C)] \times [IND_{max} - 1] + 1)$, where I is the fold of induction and C is the concentration of the test compound.

Supplemental Data

Supplemental Data include the schematic illustration of the design of the cell permeability assay; the figures for fluorescence polarization assays and luciferase assays of **SDex-CONH$_2$**, **SDex-CO-IBA**, **C-AA**, **C-AF**, **L-AA,** and **L-AF**; and the synthesis and fluorescence microscopy assays of fluorescein-labeled cyclic and linear peptides (**C1-3-FL**, **C1-4-FL**, **L1-3-FL,** and **L1-4-FL**) and are available at http://www.chembiol.com/cgi/content/full/14/6/671/DC1/.

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