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Macrocyclic Cell Penetrating Peptides: A Study of Structure-Penetration Properties

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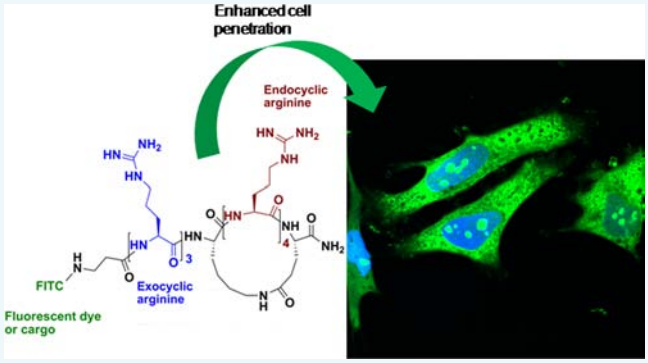
Supporting Information

ABSTRACT: Arginine-rich cell penetrating peptides are short cationic peptides able to cross biological membranes despite their peptidic character. In order to optimize their penetration properties and further elucidate their mechanisms of cellular entry, these peptides have been intensively studied for the last two decades. Although several parameters are simultaneously involved in the internalization mechanism, recent studies suggest that structural modifications influence cellular internalization. Particularly, backbone rigidification, including macrocyclization, was found to enhance proteolytic stability and cellular uptake. In the present work, we describe the synthesis of macrocyclic arginine-rich cell penetrating peptides and study their cellular uptake properties using a combination of flow cytometry and confocal microscopy. By varying ring size, site of cyclization, and stereochemistry of the arginine residues, we studied their structure−uptake relationship and showed that the mode and site of cyclization as well as the stereochemistry influence cellular uptake. This study led to the identification of a hepta-arginine macrocycle as efficient as its linear nona-arginine congener to enter cells.



INTRODUCTION

Cell-penetrating peptides (CPPs), originally known as protein transduction domains, are peptides consisting of fewer than 35 amino acids capable of penetrating cells despite their peptidic character. Initially identified as specific transduction domains embedded in protein sequences, they represent a natural mechanism of cellular targeting and entry.[1−4] Since their discovery, the cell penetrating properties of CPPs have been harnessed to internalize otherwise impermeable cargos into cells, including small molecules, macromolecules (e.g., proteins, nucleic acids), and nanoparticles.[5−11] The majority of CPPs contain a high relative abundance of positively charged amino acids such as lysine (Lys) or arginine (Arg), or display alternating patterns of polar/charged amino acids and nonpolar, hydrophobic amino acids.[9,12,13]

Arginine-rich CPPs have been used successfully to deliver a broad diversity of biologically active macromolecules intracellularly, although their mechanisms of cellular entry are still under investigation.[9,12,14−17] In the process, the guanidinium cationic groups of the Arg residues are crucial for efficient cell penetration, a role attributed to their capacity to interact with the various anion types localized on the plasma membrane such as the polar heads of phospholipids or the sulfate groups of glycosaminoglycans.[13,18,19]

Polyarginines are readily synthesized and represent the structurally simplest CPPs. They have been studied extensively for their ability to penetrate through cell membranes. It was found that cellular uptake can be achieved with oligoarginines composed of 5−15 residues.[14,20] In particular, nona-arginine (R_9) was shown to possess improved cell penetration efficiency compared to TAT peptides.[9,20] Thus, several studies have utilized R_8 and R_9 as reference tools to import into cells a variety of biological molecules including siRNA, anticancer drugs, small molecules, proteins, peptides, and oligonucleotides.[10,11]

The mechanisms by which CPPs translocate across cell membranes remain a subject of investigation. Certain CPPs, when attached to small cargos, translocate passively across the plasma membrane of cells.[21] However, an increasing number of studies have shown that cellular uptake of cationic CPPs, when conjugated to macromolecules or used at low concentrations, is temperature-sensitive, suggesting the involvement of energy-dependent processes.[17,22−25] To better delineate the mechanisms at play, Matsushita et al. recently applied siRNA library screening to identify potential partners involved in the cellular entry of polyarginine peptides. This led to the identification of

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the involvement of cotransporter gene SLC_4A_4 and the trafficking regulator gene COPA in cellular entry. Knowing that COPA plays an important role in early endosome maturation, these results suggest that cellular entry of polyarginine involves at least two steps, such as initial binding to the cell surface, followed by endosomal entry.[26]

Since polyarginines are the most commonly used CPPs, their optimization has been investigated. Replacement of (L)-Arg residues by (D) analogues and backbone modifications have produced protease-resistant analogues with improved translocation properties.[12,13] Major structural modifications aimed at improving cellular penetration and proteolytic stability, while retaining acceptable cytotoxicity, have met with various degrees of success. In particular, it has been demonstrated that increased rigidity and static display of guanidine groups is beneficial for cellular entry properties.[12,13,27−32]

In this context, macrocyclization is a well established approach to simultaneously rigidify a peptide backbone, modulate its structure−activity, improve its proteolytic stability, and reduce the entropic cost of macrocycle−target interactions.[33−36] These attributes are attractive for improving the properties of CPPs;[37−44] thus, macrocyclization and backbone rigidification of CPPs was expected to improve cellular uptake and stability, as well as to provide a means to discriminate among different cell types. Indeed, it has been proposed that guanidinium groups are forced into maximally distant positions by cyclization, increasing membrane contacts and leading to enhanced cellular penetration.[28,31,45,46]

To date, there is no systematic study of the features favorable for cellular uptake in macrocyclic polyarginines (Figure 1). In

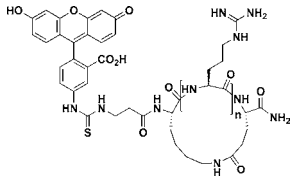

Figure 1. Representative structure of cyclic polyarginines (n = 4 and 6−9); (L) stereochemistry given as an example.

order to better understand the potential structure−penetration relationship, we synthesized several analogues in this series, with the goal of better delineating how structural variations like cyclization, stereochemistry, or endocyclic vs exocyclic display of arginine residues influence cellular uptake.

Compounds presented in this study were functionalized with a fluorescent label in the form of a fluorescein isothiocyanate (FITC) moiety. Their cellular penetration properties were assessed using flow cytometry and results were confirmed using fluorescence confocal microscopy, with the hypothesis that stereochemistry and mode of cyclization could influence cellular uptake.

RESULTS

Synthesis. Analogues were synthesized as described in Figure 2 and Supporting Information (SI) Figure S1. Briefly, synthesis relied on Fmoc chemistry and macrocyclization was performed via the formation of a lactam bridge between a Lys and a Glu residue. These two residues were previously protected as Alloc and Allyl ester, respectively, then deprotected under Pd catalysis immediately prior to cyclization. Every macrocycle was functionalized with FITC and a spacer (β-Ala, except for R_9 which was functionalized as an aminohexanoate as per previous works).

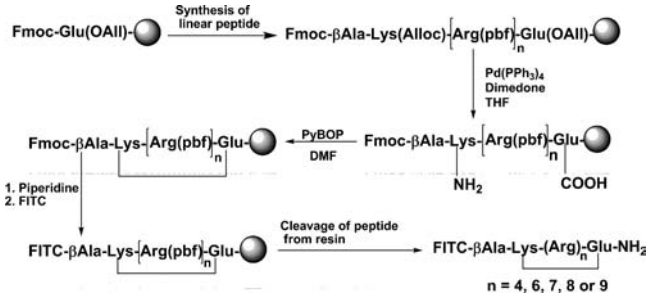

Figure 2. Synthetic scheme for the preparation of cyclic polyarginine analogues.

A total of 23 analogues (4 linear and 19 cyclic) were synthesized for this study, as summarized in Table 1. Entries 1−4

Table 1. Sequences of Linear and Macrocyclic Peptides Used in This Study[a]

no	peptides	sequences
1	R_6	FITC-βA-RRRRRR-NH_2
2	R_7	FITC-βA-RRRRRRR-NH_2
3	R_8	FITC-βA-RRRRRRRR-NH_2
4	R_9	FITC-Ahx-RRRRRRRRR-NH_2
5	Cyc-R_4	FITC-βA-[KRRRRE]-NH_2
6	Cyc-R_6	FITC-βA-[KRRRRRRE]-NH_2
7	Cyc-R_7	FITC-βA-[KRRRRRRRE]-NH_2
8	Cyc-R_8	FITC-βA-[KRRRRRRRRE]-NH_2
9	Cyc-R_9	FITC-βA-[KRRRRRRRRRE]-NH_2
10	Cyc-r_7	FITC-βA-[KrrrrrrE]-NH_2
11	Cyc-r_8	FITC-βA-[KrrrrrrrE]-NH_2
12	Cyc-r_9	FITC-βA-[KrrrrrrrrE]-NH_2
13	Cyc-(L,D)-R_6	FITC-βA-[KRrRrRrE]-NH_2
14	Cyc-(L,D)-R_7	FITC-βA-[KrRrRrRrE]-NH_2
15	Cyc-(L,D)-R_8	FITC-βA-[KRrRrRrRrE]-NH_2
16	Bicyc-0-R_6	FITC-βA-[ERRRK]-[KRRRE]-NH_2
17	Bicyc-1-R_6	FITC-βA-[ERRRK]-βA-[KRRRE]-NH_2
18	Bicyc-2-R_6	FITC-βA-[ERRRK]-(βA)$_2$-[KRRRE]-NH_2
19	Bicyc-3-R_6	FITC-βA-[ERRRK]-(βA)$_3$-[KRRRE]-NH_2
20	Cyc-R_3-R_4	FITC-βA-RRRR-[KRRRE]-NH_2
21	Cyc-R_4-R_3	FITC-βA-RRR-[KRRRRE]-NH_2
22	Cyc-R_5-R_2	FITC-βA-RR-[KRRRRRE]-NH_2
23	Cyc-R_6-R_1	FITC-βA-R-[KRRRRRRE]-NH_2

[a]Lower case letters indicate (D) and upper case letters (L) stereochemistry. Residues between brackets are included in the macrocycle, closed by macrolactamization between the Glu (E) and Lys (K) side chains.

(Table 1) are reference compounds aimed at comparing cellular uptake with published results; entries 5−15 are side-chain to side-chain macrocycles containing 4−9 homochiral (L)Arg or (D)Arg residues or heterochiral (L,D)Arg residues. It should be kept in mind that between homo- and heterochiral macrocycles, the projection of side chains with respect to the plane of the macrocycle differs. Entries 16−19 are bicyclic macrocycles built as described in SI Figure S1, with the goal of assessing the influence of additional rigidification on cellular uptake; finally, entries 20−23 are macrocyclic polyarginines (3−6 residues) bearing 1−4 exocyclic arginine residues built to assess the influence of endocyclic vs exocyclic residues on cellular uptake.

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Compounds were synthesized on solid phase synthesis and purified to >95% purity (UPLC-UV).

The cellular uptake of new compounds was first assessed by flow cytometry, then confirmed using confocal microscopy as described below.

Flow Cytometry. Flow cytometry analysis was used to quantify the cellular uptake of the new constructs in HeLa cells. Uptake measurements were based on changes in the mean cellular fluorescence following incubation with FITC-labeled peptides, relatively to linear control R_9. Knowing that conventional flow cytometry cannot distinguish between intracellular and cell surface fluorescence, trypsin treatment was applied (Trypsin-EDTA 0.05%, 5 min) to remove cell surface-bound peptides prior to analysis. The efficiency of trypsin treatment for macrocycles was confirmed since treatment of Cyc-R_4, which contains only 4 Arg residues, showed a very weak emission signal comparable to the signal of autofluorescence, confirming that membrane-bound cyclic peptides were indeed detached from the cell surface (Figure 3B). Additionally, it was confirmed that the uptake of Cyc-R_9 is concentration-dependent (Supporting Information, Figure S2).

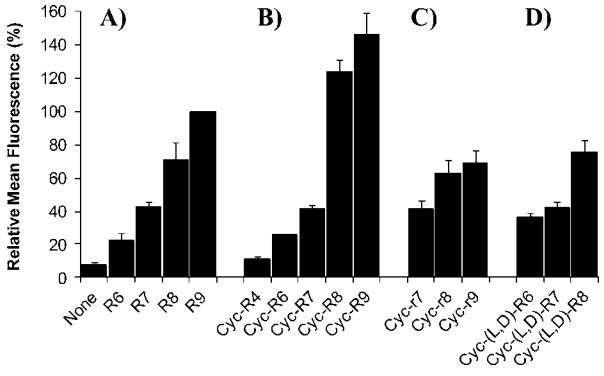

Figure 3. Flow cytometry analysis of HeLa cells treated with (A) linear reference compounds; (B) all (L) macrocycles; (C) all (D) macrocycles; and (D) alternating (L,D) macrocycles. Each value represents the average of three experiments, and values are normalized with respect to control compound R_9.

Linear peptides R_6−R_9 were used as reference compounds and demonstrated an increasing uptake with increasing arginine contents (Figure 3A), consistently with previous literature reports.[14] In order to better understand the influence of structural variations on cellular uptake of macrocyclic constructs, we next analyzed the uptake of the cyclic analogues of the polyarginine peptides (Figure 3B). Cyc-R_4 and Cyc-R_6 showed a very weak emission signal (commensurate with the autofluorescence signal), suggesting little or no uptake. A significant increase in uptake was noted for Cyc-R_8 and Cyc-R_9. Importantly, Cyc-R_9 displayed 40% higher uptake than its linear analogue R_9.

In order to better understand the influence of stereochemistry on internalization by HeLa cells, we prepared both homochiral (L) macrocycles, homochiral (D) macrocycles, as well as heterochiral (L,D) macrocycles, then compared their uptake by flow cytometry (Figure 3B−D). It is interesting to note that cycloocta-arginine macrocycles were particularly sensitive to the influence of stereochemistry, as opposed to their cycloheptaarginine counterparts. Indeed, homochiral (L) analogue Cyc-R_8 displayed higher levels of uptake (125% the value obtained for linear R_9) than analogues Cyc-r_8 and Cyc-(L,D)-R_8 (63% and 75% the value reached by R_9, respectively). In fact, Cyc-R_8 demonstrated improved penetration compared to the standard linear R_9 peptide. Similar observations were made for cyclononaarginines. For example, Cyc-R_9 was internalized twice as much as Cyc-r_9 and 50% more than its linear analogue. Finally, Cyc-R_9 macrocycle displayed an improved uptake compared to all the other cyclic or linear analogues (Figure 3B).

To confirm that the fluorescence observed in flow cytometry is intracellular and not associated with cell surface, uptake experiments of R_9, Cyc-R_9, and Cyc-r_9 were repeated in the presence of Trypan Blue to quench extracellular fluorescence (Figure 4, white vs black bars).[47] No major loss of fluorescence

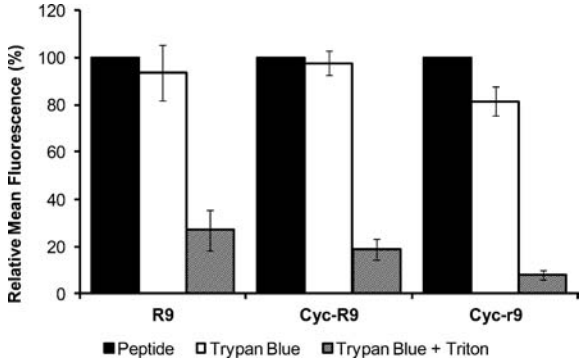

Figure 4. Flow cytometry analysis of HeLa cells treated with R_9, Cyc-R_9, and Cyc-r_9 peptides in the presence and absence of Trypan blue and Triton-X100. Each value represents the average of three experiments.

was observed in the presence of Trypan Blue in the case of R_9 and Cyc-R_9, indicating that the observed fluorescence is indeed intracellular. In the case of Cyc-r_9, 20% of the fluorescence was quenched in the presence of Trypan Blue, suggesting that a small portion of the compounds remain membrane-bound. However, when Triton-X100 was added to permeabilize the cells, extensive quenching was observed, as expected if the fluorescence is intracellular (Figure 4, gray bars).

In order to better understand how further rigidification influences cellular uptake, we next explored the effect of double cyclization, spacing between the two macrocycles, and the respective contribution of macrocyclic and linear groups on cellular uptake. This was performed on analogues bearing 6 Arg derivatives, to assess whether increased structural rigidification could bring these analogues to levels of uptake similar to those observed with linear R_8 or R_9 (see Table 1 for structures). Indeed, it was demonstrated that rigid scaffolds bearing as little as 4 or 5 guanidine groups could be as efficient as linear R_9 to mediate cellular uptake.[27−29,31,32] Macrobicyclic hexa-arginine derivatives were synthesized using a similar approach to that of macrocycles with a single ring (see SI Figure S1). In some cases, spacers composed of 1−3 βAla residues were incorporated between the two rings, in order to introduce some flexibility to the peptide and potentially impart some α-helical contents.[48] For comparison, we present in Figure 5A the results obtained by flow cytometry of Cyc-R_6 and the different bicyclic peptides bearing 6 Arg residues. One can observe that in the whole series of hexa-arginine derivatives, the mode of cyclization and the presence of a linker had little influence on cellular uptake, which remained low in all cases. Bicyclization of hexa-arginine had either no effect, or a small tendency toward a negative effect.

We next investigated the respective impact of endo- vs exocyclic Arg residues on cellular uptake. Given the poor results

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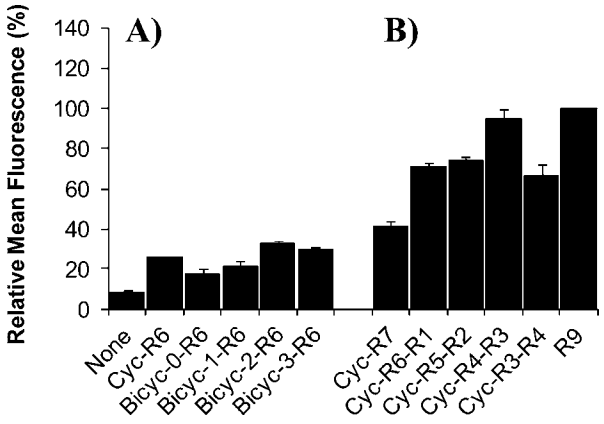

Figure 5. Flow cytometry analysis of HeLa cells treated with (A) bicyclic and (B) 7 arginine monocyclic peptides. Each value represents an average of three experiments, and values are normalized with respect to control compound R_9.

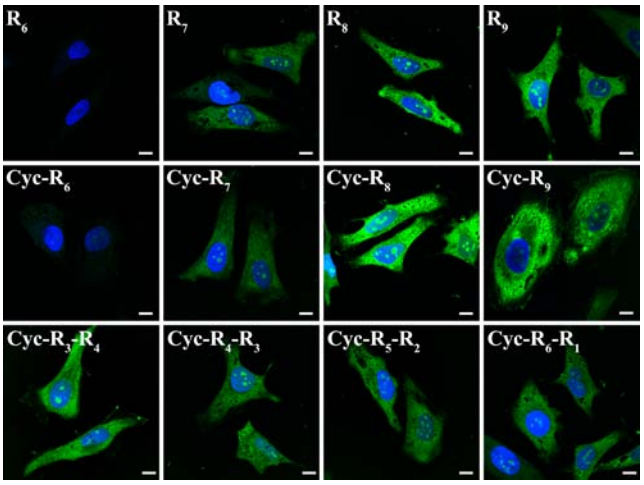

Figure 6. Confocal microscopy images of HeLa cells treated with FITC-labeled linear and macrocyclic peptides (in green). Nuclei are marked in blue. Scale bar, 10 μm.

obtained in the previous hexa-arginine bicyclic derivatives, this was performed on hepta-arginine derivatives (Figure 5B). In this series, we compared a macrocycle containing 7 endocyclic Arg groups (Cyc-R_7) to analogues bearing 6 endocyclic and 1 exocyclic Arg (Cyc-R_6-R_1), 5 endocyclic and 2 exocyclic Arg (Cyc-R_5-R_2), 4 endocyclic and 3 exocyclic Arg (Cyc-R_4-R_3), and finally 3 endocyclic and 4 exocyclic Arg (Cyc-R_3-R_4) groups. Thus, the number of Arg residues was kept constant in this series. Flow cytometry results (Figure 5B) confirmed the possibility to significantly increase cellular uptake via the structural modification of these peptides. The optimal structure, Cyc-R_4-R_3, contains 4 endocyclic and 3 exocyclic Arg residues. Ultimately, the latter reached the same level of internalization as the standard linear R_9 derivative, despite the fact that it possesses 7 instead of 9 Arg residues. In the linear series (Figure 3A), the internalization of linear R_7 was ~40% of that of linear R_9. These results were consistent with those observed in confocal microscopy (see below).

Confocal Microscopy. The cellular uptake of selected linear and macrocyclic peptides was subsequently assessed by confocal microscopy on HeLa cells. Toward this end, cells were incubated with compounds (5 μM) for 30 min at 37 °C, then imaged by confocal microscopy after fixation on coverslips, or using live cells. The images of fixed HeLa cells treated with linear peptides R_6–R_9 are shown in Figure 6 (top panels) as controls. Cells treated with linear R_6 polyarginine showed little, if any, intracellular fluorescence, while treatment with R_7–R_9 showed increasing levels of fluorescence with increasing number of Arg residues under the same imaging conditions, as previously reported.[14]

Similarly to linear peptides, internalization of macrocyclic peptides showed strongly diffused signals for peptides containing 7, 8, and 9 arginine residues (Cyc-R_7, Cyc-R_8, Cyc-R_9), consistent with a predominantly cytoplasmic distribution (Figure 6, middle panels). As observed with linear peptides, increasing the number of Arg residues was associated with increased uptake from Cyc-R_6 to Cyc-R_8. However, there appeared to be no significant increase between Cyc-R_8 to Cyc-R_9 (Figure 6, middle panels). In parallel, the macrocyclic peptides possessing an exocyclic arginine chain showed significant variations in cellular uptake, whereby Cyc-R_3-R_4 displayed a stronger diffused signal (Figure 6, bottom panels) compared with the other peptides of this family (Cyc-R_4-R_3, Cyc-R_5-R_2, Cyc-R_6-R_1).

The results of internalization of Cyc-r_7, Cyc-r_8, and Cyc-r_9, as well as those of Cyc-($_{L,D}$)-R_6, Cyc-($_{L,D}$)-R_7, and Cyc-($_{L,D}$)-R_9, were also confirmed by confocal microscopy (SI Figures S3, S4).

Selected linear and macrocyclic peptides were also tested using live cell imaging (SI Figure S5), given that fixation may lead to artifactual cell permeabilization and uptake.[49] Essentially, results obtained with live cell imaging were consistent with those obtained with fixed cells, indicating that fixation did not alter cellular penetration.

DISCUSSION

The aim of this study was to better understand how the mode of macrocyclization, the stereochemistry, and the endo vs exocyclic display of Arg in macrocyclic polyarginines influence cellular uptake. Indeed, it was previously reported, on one hand, that a minimal number of 8–9 arginine residues is required for efficient cell entry of linear arginine-rich peptides, yet it is also known that structural rigidification of the molecule could improve cellular penetration and allow a reduction in the number of exposed guanidine motifs, down to 4 or 5 guanidine units.[27−29,31,32] Flow cytometry was used to quantify the extent of cellular uptake in the various series, and confocal microscopy was used to confirm uptake and assess preliminarily the intracellular distribution. It should be kept in mind that the only charged units in these peptides are the guanidinium groups since Rink amide resin was used to deliver C-terminal amide peptides.

First, results from flow cytometry of the control linear peptides confirmed that increasing the number of arginine increased cell internalization by 20% to 30% per arginine residue, as previously reported (Figure 3A).[14]

We further asked how structural modifications to the macrocycles could influence cellular uptake. Indeed, reported molecular modeling suggested that a subset of the side chain guanidinium groups of these transporters might be required for transport involving contact with a common surface such as a plasma membrane or cell surface receptor, suggesting the possibility to develop the structure–uptake relationship and possibly cellular specificity.[27−29,31,46,50] Spacers such as glycine have been introduced between arginine amino acids, showing that the spacing between the arginine residues in a set of linear peptides could influence uptake.[50] Consequently, by cyclizing polyarginine peptides, we expected to increase the distance

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between the guanidinium groups by way of conformational restrictions, without the addition of spacer elements and increase in molecular weight. Uptake measurements using flow cytometry on monocyclic peptides (Figure 3B) showed a dramatic effect on the cellular uptake of octa- and nona-arginine derivatives. The cyclic form of octa-arginine was not only able to penetrate into cells more efficiently than the linear form, it also exhibited superior uptake than control linear nona-arginine. This result is in agreement with literature describing the cyclic arginine-rich cell-penetrating peptides showing enhanced cellular uptake relative to their linear and more flexible counterpart.[31,38] It has been proposed that guanidinium groups are forced into maximally distant positions by cyclization and this orientation increases membrane contacts leading to enhanced cell penetration. Interestingly, macrocyclic analogues of (L)-Arg gave much higher penetration than those of (D)-Arg congeners or those alternating (L) and (D) stereochemistry (Figure 3B–D). This result is in agreement with the works of Verdurmen et al., who reported that cationic (L)-cell-penetrating peptides are taken up more efficiently than their (D)-counterparts in MC57 fibrosarcoma and HeLa cells but not in Jurkat T leukemia cells.[46]

To further rigidify structure and improve cellular uptake, we examined bicyclic hexa-arginine analogues (Figure 5). Despite increased structural rigidification, cellular uptake of these variously double-cyclized analogues remained very low (Figure 5A).

Subsequently, the position of cyclization within the macrocycle was varied. Based on the disappointing results obtained in the hexa-arginine series, we focused this part of the study on hepta-arginine derivatives. Thus, hepta-arginine macrocycles containing 7, 6, 5, 4, or 3 endocyclic and 0, 1, 2, 3, or 4 exocyclic Arg residues, respectively, were synthesized (Table 1). Flow cytometry data showed an important effect of changing the position of macrocyclization on cellular uptake. Indeed, all the hepta-arginine cyclic peptides with exocyclic arginine residues possessed enhanced cellular internalization compared to Cyc-R_7 with 7 endocyclic Arg residues. Interestingly, the cyclic form Cyc-R_4-R_3 was able to penetrate cells more efficiently than the other hepta-arginine derivatives, and possessed an uptake comparable to that displayed by linear nona-arginine (Figure 5B). This important result confirms that structural modification of the peptide enhances cellular uptake per Arg residue in this macrocyclic series; however, a clear relationship remains to be identified. We performed circular dichroism analyses of the peptides presented in this manuscript (SI Figures S6–S8); however, they did not lead to any conclusive relationship between apparent secondary structure and cellular uptake.

Results from confocal microscopy performed on fixed or live cells (Figure 6 and SI Figures S3–S5) were similar and demonstrated qualitatively that 7 Arg residues are necessary to reach a decent level of uptake.

CONCLUSION

We have synthesized diversely cyclized macrocyclic oligoarginine analogues in order to better understand the influence of conformational restrictions on cellular penetration in HeLa cells. Similarly to linear peptides, our results showed that increasing arginine content in the macrocycles enhanced cellular uptake. We also demonstrated that incorporation of 6 arginines in bicyclic peptides was not sufficient to enhance cellular uptake. Finally, we explored the effect of stereochemistry and mode of cyclization and provided the evidence of an important effect on cellular uptake. In this series, the most efficient macrocycle contained 4 endocyclic and 3 exocyclic Arg residues. We are currently investigating additional macrocyclic analogues of the hepta-arginine family, as well as further details on the mechanisms of cellular uptake and the potential of this new macrocycle to mediate the cellular uptake of functional cargos.

EXPERIMENTAL SECTION

Peptide Synthesis. Linear and cyclic peptides were synthesized using standard solid-phase synthesis with Fmoc chemistry on a Tribute peptide synthesizer (Protein Technologies Inc.) with IR activation. The protected amino acids Fmoc-L-Arg(Pbf)-OH, Fmoc-D-Arg(Pbf)-OH, Fmoc-L-Glu-(OAll)-OH, Fmoc-L-Lys(Alloc)-OH, and Fmoc-βAla-OH were purchased from ChemImpex International and used with no further purification. The protected peptide resins used to synthesize the macrocycles were prepared using 0.25 g of Rink amide resin from Rapp Polymere (0.23 mmol of NH_2/g of resin) by first coupling to the resin, previously deprotected with piperidine/DMF (1:1). Couplings were performed using HATU (1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]-pyridinium 3-oxide hexafluorophosphate), using ~20 min for all amino acids as monitored by UV. Upon complete formation of the protected linear peptide, resins were washed with dimethylformamide (DMF), isopropanol, and dichloromethane (DCM) and dried in vacuo. At this step, the Alloc and Allyl protecting groups of Lys and Glu residues were simultaneously removed. A solution of dimedone (15 equiv) in 10 mL dry tetrahydrofuran (THF) was added under argon to the resin. Subsequently, 0.45 equiv of $Pd(PPh_3)_4$ was added to the resin and the reaction was mechanically stirred for 3 h under Ar. Then, the resin was washed with DMF, isopropanol, and DCM.

To perform macrocyclization, the resin was suspended in 20 mL DMF, followed by side-chain to side-chain cyclization of the peptide by addition of PYBOP (Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate, 10 equiv), and DIPEA (4 equiv) overnight. The last step of synthesis was to attach the FITC fluorophore by deprotecting the peptide resin with piperidine/DMF (1:1), followed by reaction with FITC in DCM/Pyridine (7:3) during 30 min. Because FITC conjugates are susceptible to Edman degradation, we used β-alanine as a spacer.[51] The bicyclic peptides were prepared using the standard solid phase method described in SI Figure S1.

Peptides were cleaved from the resin and the Pbf protecting groups were simultaneously removed using a mixture of TFA/TIPS/H_2O (95:2.5:2.5) for 5 h. The resin was filtered, washed with 1 mL of the cleavage solution, and then the crude peptide recovered by precipitation with cold diethyl ether to give an orange powder that was purified by preparative HPLC (Waters Autosampler 2707, Quaternary gradient module 2535, UV detector 2489, fraction collector WFCIII) equipped with an ACE5 C18 column (250 × 21.2 mm, 5 μm spherical particle size) and water + 0.1% TFA and acetonitrile as eluents. The purification was monitored at 245 nm and the fractions corresponding to the major peak were collected, pooled, and lyophilized. Purities and molecular weights of the corresponding peptides were determined using an Acquity H-Class UPLC-MS system with PDA UV and SQD2Mass detectors equipped with a C18 column (2.1 × 50 mm, 1.7 μm spherical particle size column). All peptides possessed UV purity >92% (for characterization details, please refer to Supporting Information).

Confocal Microscopy. HeLa cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Approximately 5 × 10^4 cells were plated on coverslips in 35

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mm culture dishes. The cells were grown for 24 h in Dulbecco's modified Eagle's high glucose medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT, USA), and 1% Penicillin−Streptomycin−Glutamine solution (Invitrogen, Carlsbad, CA, USA). The day of the experiment, cells were treated by adding 5 μM FITC-labeled peptide to the culture medium and incubated for 30 min at 37 °C in the presence of 5% CO_2. Cells were then washed twice with phosphate buffered saline (PBS) and fixed for 30 min in 3% paraformaldehyde (PFA) in 100 mM phosphate buffer, pH 7.4. PFA was quenched for 10 min using a solution of 50 mM ammonium chloride and nuclei were marked using 1 μg/mL Hoechst 33342 stain for 10 min. The specimens mounted on slides using mounting medium (1% *n*-propyl gallate in glycerol/PBS (1:1)) and visualized using an inverted confocal laser-scanning microscope (FV1000, Olympus, Tokyo, Japan) equipped with a PlanApo 60×/1.42 oil immersion objective (Olympus, Tokyo, Japan). *Olympus Fluoview* software version 1.6a was used for image acquisition and analysis. The images were further processed using *Adobe Photoshop* (Adobe Systems, San Jose, CA, USA). FITC-labeled peptides and nuclei were excited at 488 and 405 nm and detected with 500−600 and 425−475 nm band-pass filters, respectively.

Flow Cytometry. Approximately 5×10^4 HeLa cells were plated on 35 mm culture dishes. After 48 h, cells were treated with the FITC-labeled peptides (5 mM) as described above. Cells were washed once with PBS and taken off using Trypsin-EDTA 0.05% (GIBCO, Invitrogen, Carlsbad, CA, USA). Finally, cells were resuspended in PBS buffer containing 10 mg/mL propidium iodide (PI) to establish the live gate to exclude debris and dead cells. A minimum of 10 000 gated events by sample were acquired and analyzed by a FACScan cytometer (Becton Dickinson, Mountain View, CA) equipped with a 15 mW argon ion laser tuned at 488 nm. The emitted fluorescences were split and collected as follows: FITC 530 ± 15 nm (green), PI 585 ± 21 nm (orange).

Flow cytometry analyses were performed in the presence of Trypan Blue to ensure extracellular fluorescence quenching. Cells were treated with peptides in the conditions described above. Signal was then acquired a first time in cytometry. Cells were then incubated with Trypan Blue (0.05% w/v in PBS) for 3 min before the second signal acquisition. Finally, the same cells were permeabilized with 0.05% (v/v) Triton-X100 and fluorescence was measured one last time by cytometry.

For live cell experiments, cells were cultured and treated with peptides as described above. The coverslips were mounted directly onto slides using mounting medium and visualized within the next 10 min.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthetic approach for bicyclic peptides, additional confocal microscopy results, table of characterization, UPLC and MS results. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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