Supramolecular trap for catching polyamines in cells as an anti-tumor strategy

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Polyamines are essential for the growth of eukaryotic cells and can be dysregulated in tumors. Here we describe a strategy to deplete polyamines through host-guest encapsulation using a peptide-pillar[5]arene conjugate (P1P5A, P1 = RGDSK(N3)EEEE) as a supramolecular trap. The RGD in the peptide sequence allows the molecule to bind to integrin αvβ3-over-expressing tumor cells. The negative charged glutamic acid residues enhance the inclusion affinities between the pillar[5]arene and cationic polyamines via electrostatic interactions and facilitate the solubility of the conjugate in aqueous media. The trap P1P5A efficiently encapsulates polyamines with association constants of 10⁵–10⁶ M⁻¹. We show that P1P5A has a wide spectrum of antitumor activities, and induces apoptosis via affecting the polyamine biosynthetic pathway. Experiments in vivo show that P1P5A effectively inhibits the growth of breast adenocarcinoma xenografts in female nude mice. This work reveals an approach for suppressing tumor growth by using supramolecular macrocycles to trap polyamines in tumor cells.
Polyamines, mainly including the aliphatic cations spermine (spm), spermidine (spd), and putrescine (put), exist in most living organisms and regulate a series of physiological processes including cell proliferation and differentiation. Maintaining polyamine homeostasis by synergistic action between biosynthesis, catabolism and transport, is critical to cell survival. Rapidly dividing cells like tumor cells require high amounts of polyamines to sustain tumor-related division. Therefore, depleting polyamines to induce apoptosis of tumor cells has been identified as a feasible approach for the development of antitumor agents. The construction of inhibitors targeting the polyamine biosynthetic pathway is one strategy that has been extensively studied. The most representative inhibitor is 2-difuromethylornithine (DMFO), which has been extended to Phase I–III clinical trials. Unfortunately, DMFO lacks clinical efficacy as a single agent in multiple clinical trials owing to its poor transport into cells and intracellular compensatory mechanisms. Another methodology that has been reported is the use of polyamine analogs that are disparate enough from natural polyamines so that they can regulate physiological function, but not so different that they are not overly toxic to normal cells. However, it is very difficult to predict and control structural properties that favor both bioactivity and safety.

Herein, we propose a strategy for tumor treatment, in which polyamines are excessively depleted through host–guest encapsulation. Pillar[5]arenes, an emerging type of macrocyclic hosts with a unique rigid and intrinsic symmetrical structures, has an outstanding ability to bind various kinds of guest molecules in a selective manner. With a diameter of ~4.7 Å, pillar[5]arene is an ideal host for linear spm, spd, and put due to their good water solubility, the host–guest mixture was precipitated, indicating their strong interactions. No precipitate was found when mixing the three polyamines with carboxylatopillar[5]arene (CP5A), a widely used water-soluble pillar[5]arene. Figure 2a–c shows the spectra of spm in D2O recorded in the absence and presence of one equivalent of CP5A at 298 K. The proton signals of spm, especially for the middle methylenes Hd and He, underwent substantial upfield shifts (Δδ = −1.53 ppm for H3 and H4, respectively) and experienced considerable broadening upon the addition of CP5A. When the spectra were recorded above 313 K, a reduction in the spectral broadening of protons H4 and H5 was observed (Supplementary Fig. 18). Such spectral differences, which reflect complexation-induced shielding effects, are consistent with the formation of an inclusion complex and indicate that the middle methylene part (H3 and H4) is located in the cavity of pillar[5]arene. In addition, the peaks for the CP5A host shifted downfield due to complexation-induced deshielding effects. From 2D NOESY analysis, NOE correlations were observed between H3, H4, H5, and H6 of spm and the aromatic protons H1 of CP5A, as well as those between methylenes H2 located at the cavity portals of the host and the outer protons H3, H4, and H5 of spm (Supplementary Fig. 19). Both the 1H NMR and 2D NOESY results confirmed the formation of inclusion structures. Similar
Cytotoxicity of P1P5A. To examine the effect of P1P5A on cell viability, several tumor and normal cell lines were treated with various concentrations of P1P5A for 48 h, and then the cell viability was measured using a Cell Counting Kit-8 (CCK-8) assay. Concentration-dependent cell death was observed for all cell lines with the half-maximum inhibitory concentration (IC₅₀) values ranging from 8 to 53 μM (Table 1). It was hypothesized that the capture of polyamines in an inert cavity of pillar[5]arene would disturb their normal physiological effects, and that excessive depletion of polyamines would cause cell death. No cytotoxic activity was observed for peptide P1, CP5A, or a 1:1 physical mixture of them even up to concentrations of 100 μM (Supplementary Fig. 27), indicating that neither the peptide nor pillar [5]arene alone was responsible for the cytotoxicity and that the RGD sequence greatly promoted the trapping of P1P5A into the cells. To further confirm this finding, two control conjugates (P2P5A and P3P5A), in which the RGD sequence was replaced by GGD and RGE, respectively (see Supplementary Figs. 9, 12–15 and Supplementary Table 1), were synthesized. As expected, they did not effectively inhibit cell growth in any cell lines (Table 1, Supplementary Fig. 27). It was reasonable that GGD and RGE could not effectively increase the conjugate uptake. The RGD peptide sequence, as an essential and highly conserved component, has been demonstrated to bind with high affinity to integrin αvβ₃, thus facilitating the entry of the conjugate into cells better than the GGD or RGE sequence.

Induction of apoptosis. Previous studies have shown that dysregulation of polyamine homeostasis can lead to apoptosis. In order to determine whether the synthetic supramolecular trap can induce programmed cell death or necrosis, the FITC-Annexin V/propidium iodide (PI) method was employed in the human breast adenocarcinoma cell line MCF-7. As shown in Fig. 3a, after incubation for 48 h, negligible apoptosis happened in the PBS group. However, the percentage of apoptotic cells (including early and late apoptotic cells) in the group treated with 25 μM P1P5A was about 46.7%, while that in the presence of 50 μM P1P5A was significantly higher at 73.0% (Fig. 3b, c). These results clearly indicate that P1P5A could effectively induce the apoptosis of tumor cells and that the apoptosis rate depended on the dosage. Moreover, when seven other cell lines were treated with P1P5A, similar regulation of cell apoptosis was observed (Supplementary Fig. 28).

To further prove the occurrence of apoptosis, a commercially available terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) kit was used to observe cells treated with different doses of P1P5A. The TUNEL staining assay displayed that P1P5A could induce severe apoptosis of MCF-7 cells (green dots) in both dosage groups (Fig. 3e, f) compared with almost no apoptosis in the control group (Fig. 3d). These results were in

Table 1 IC₅₀ values of P1P5A against selected cell lines (μM)

| Tumor types             | Cell lines | IC₅₀ (μM) | P1P5A | P2P5A | P3P5A |
|------------------------|------------|----------|-------|-------|-------|
| Breast cancer          | MCF-7      | 18.40    | 92.06 | 58.69 |
| Glioma                 | C6         | 30.16    | >100  | >100  |
|                        | U87        | 22.53    | >100  | 73.43 |
| Neurona                 | Neuro-2a   | 8.52     | 79.75 | 45.07 |
| Lung cancer            | A549       | 34.81    | >100  | >100  |
| Hepatocellular carcinoma | HepG-2    | 30.85    | >100  | >100  |
| Normal                 | LO2        | 49.17    | >100  | >100  |
|                        | HUVEC      | 52.15    | >100  | >100  |
accordance with the flow cytometry results, revealing that P1P5A effectively inhibited tumor cell growth by inducing apoptosis.

Furthermore, transmission electron microscopy (TEM) was used to directly view the ultramorphological changes of MCF-7 cells treated with P1P5A. As shown in Fig. 3h, i, the cells treated with P1P5A were apoptotic with a changed morphology, condensed chromatin, and damaged or disappeared organelles; while the control cells presented a normal structure (Fig. 3g). The apoptotic process started with the condensation of chromatin at the nuclear periphery, then many blebs were generated, and finally swollen or hollow mitochondria emerged (Fig. 3i).

Molecular mechanism. Confocal laser scanning microscopy (CLSM) was conducted to verify the distribution of the supramolecular traps in cells. 7-Fluoro-4-nitro-2,1,3-benzoxadiazole (NBD-F), a commercially available fluorescent labeling reagent (green fluorescence), was used to label P1P5A via the amino group of the additional lysine residue at the C-terminus to obtain the conjugate P4P5A (Supplementary Figs. 9, 16, 17, and Supplementary Table 1). As shown in Fig. 4a, 4',6-diamidino-2-phenylindole (DAPI) was used to stain the cell nucleus blue; and 1,1'-dioctadecyl-3,3',3'-tetramethylindodicarbocyanine (DiD), a lipophilic fluorescent dye, was used to stain cell membranes and other fat-soluble biological structures red. MCF-7 cells exhibited strong intracellular green fluorescence after incubation with 50 μM P4P5A for 48 h, and green dots corresponding to P4P5A colocalized with DiD, revealing an obvious internalizing behavior and concentrated distribution in the cytoplasm. The above results showed that the conjugate was mainly enriched in the cytoplasm and did not enter the nuclei of MCF-7 cells.

Having confirmed the distribution of the conjugates in the cytoplasm, we evaluated the effect of P1P5A on intracellular
polyamine depletion. As shown in Fig. 4b, P1P5A treatment for 6 h did not change the intracellular concentration of spm, spd, or put in MCF-7 cells. This result was quite reasonable because the host-guest complex did not change the polyamine structures. Meanwhile, by western blot analysis, we measured the contents of ornithine decarboxylase (ODC), spermidine synthase (SPDS), and spermine synthase (SPMS) which generate the corresponding polyamines. The first rate-limiting step of polyamine biosynthesis is that ornithine generates put catalyzed by ODC. SPDS and SPMS, specific aminopropyl transferases, sequentially produce spd from put and spm from spd, respectively (see Supplementary Fig. 29). When the P1P5A traps catch these polyamines to form the host-guest complexes, more enzymes would be expressed to assist the production of more spm, spd, and put as feedbacks. As shown in Fig. 4c, the expression levels of ODC in cells treated with P1P5A were much higher than those treated with PBS, P1, CP5A, and 1:1 P1 + CP5A. The gray-scale results display a dose-dependent effect between the low- and high-dose groups. The contents of ODC reflected the consumption of put due to compensatory mechanism. In contrast, treatment with P1P5A did not induce increase in expression of SPDS and SPMS, which is reasonable since SPDS and SPMS are constitutively expressed and are not easily induced by external factors. The effect of host-guest interaction, although did not change the chemical structure of these polyamine molecules, merely affected the normal physiological activities of spm, spd, and put through inclusion in the host cavity.

**In vivo tumor inhibition studies.** To assess the efficacy of P1P5A in vivo, subcutaneous tumor xenograft mouse models were used. The results (Fig. 4a, b) revealed that the tumor volumes in the PBS, P1, CP5A, and 1:1 mixture of P1 and CP5A groups increased about sixfold over the original state after 10 days, while both P1P5A groups, 100 mg kg$^{-1}$ (L) and 200 mg kg$^{-1}$ (H) per time, exhibited a remarkable inhibitory effect on tumor growth. The tumor volume was much less in the high-dose P1P5A group than in the low-dose group. The final tumor weight was measured to further validate the efficacy of P1P5A (Fig. 5c). The normalized tumor weight of the PBS group (0.87 ± 0.14 g) was 93% higher than that of the low-dose P1P5A group (0.45 ± 0.19 g) and 260% higher than that of the high-dose group (0.24 ± 0.14 g). The 1:1 mixture of P1 and CP5A had no antitumor activity. In addition, we also tried three lower doses of P1P5A, 25, 50, and 75 mg kg$^{-1}$. Although the P1P5A groups of 25 and 50 mg kg$^{-1}$ had no obvious inhibitory effect on tumor growth, the 75 mg kg$^{-1}$ group embodied certain antitumor activity (Supplementary Fig. 30). Taken together, these results indicated that P1P5A could efficiently suppress tumor growth and that the covalent linkage of the peptide and pillar[5]arene macrocycle played a decisive role in the antitumor activity.

In addition to evaluating the therapeutic effect with different treatments, hematoxylin and eosin (H&E), TUNEL, and CD31 staining assays were performed to detect apoptotic cells and inhibition of angiogenesis in tumor tissue (Fig. 5f). The images of H&E-stained tumor tissues showed the lack of discernible boundary regions and the absence of many nuclei, indicating that the two doses of P1P5A could induce necrosis. Compared to the other groups, the P1P5A groups showed the highest number of TUNEL-positive tumor cells, indicating massive apoptosis. In addition, the CD31-stained immunohistochemical sections of the tumors showed that the P1P5A groups had the least angiogenesis, confirming that the treatment efficiency inhibited cell proliferation in the tumor tissues.

For antitumor therapeutic agents, safety in vivo is very important. Therefore, changes in body weight and the survival rate of the tumor-bearing mice after treatment were determined to reflect the systematic toxicity. As shown in Fig. 5d, the body weight did not decrease significantly compared to the PBS group. The body weight changes were not significantly different among all groups except for the high-dose P1P5A group, which showed a slight decrease in body weight. The result suggests that the systemic toxicity of P1P5A was not dose-dependent and could be a potential treatment for advanced tumors.
weights of the mice in all groups remained almost the same as that of the control group, demonstrating that P1P5A showed a negligible effect on body weight. In addition, Kaplan–Meier analyses showed that the median survival of the mice treated with PBS was 24 days; for P1P5A, it was significantly prolonged to over 36 days (Fig. 5e). To further prove the low systemic toxicity of P1P5A for tumor therapy, the main organs including the heart, liver, spleen, lung, and kidney of the mice with different treatments were harvested and analyzed by H&E staining at 10 days post operation. The results of the histological analysis showed no significant inflammatory or toxicological responses in the control groups (P1, CP5A, 1:1 P1 + CP5A) or the P1P5A administration groups, compared to the PBS group (Supplementary Fig. 31). These results indicate that P1P5A was well-tolerated in the major organs.

Discussion

In summary, we have described a supramolecular therapeutic agent P1P5A as an inhibitor of tumor growth. The pillar[5]arene molecular container could trap polyamines, restrict their normal physiological effects, and therefore induces cell apoptosis. Particularly, the peptide segment containing the RGD sequence played a crucial role in the activity of the compound, due to its efficient binding to integrin α6β1 and mediation of specific endocytosis. P1P5A could inhibit several kinds of tumor cells via a polyamine-dependent pathway in in vitro experiments. In addition, in vivo experiments also confirmed the antitumor efficiency of P1P5A that it effectively inhibited the growth of MCF-7 xenografts in female nude mice, together with a high median survival and a low systemic toxicity. Overall, this work proposed a strategy that macrocyclic derivatives can perform as antitumor agents instead of only as supramolecular vehicles for drug delivery. This research might pave a way to win the battle against cancer.

Methods

General. All reagents were purchased commercially and used without further purification unless otherwise noted. CP5A was synthesized and purified according to previous reports. N-Fluorenyl-9-methoxy carbonyl (Fmoc)-protected t-amino acids were purchased from GL Biochem Ltd. (Shanghai, China). Ring amide resin was purchased from Nankai Hecheng (Tianjin, China). Roswell Park Memorial Institute (RPMI) 1640 medium, minimum Eagle’s medium (MEM), and Dulbecco’s modified Eagle medium (DMEM) were purchased from Gibco (Thermo Fisher Scientific). Fetal bovine serum (FBS), penicillin–streptomycin and PBS were purchased from Invitrogen (Carlsbad, CA, USA). The Cell Counting Kit-8 (CCK-8) was purchased from Dojindo China Co. Ltd. (Shanghai, China). The Annexin V-FITC/PI apoptosis detection kit was purchased from Zoman Biotechnology Co. Ltd. (Beijing, China). MCF-7, C6, U87, Neuro-2a, A549, HepG2, LO2, and HUVEC were purchased from Cell Resource Center (Beijing, China). Anti-ODC antibody (ab660067), anti-spermidine synthase antibody (ab111884), and anti-spermine synthase antibody (ab156879) were all purchased from Abcam Co. Ltd. (Shanghai, China). 1H NMR, 13C NMR, and 2D NOESY spectra were recorded using a JNM-ECA-400 or a Bruker Advance 500 MHz spectrometer. High-resolution mass spectra were recorded on a Bruker Daltonics, Inc. APEX III 7.0 TESLA FTMS instrument and a Bruker Daltonics, Inc. APEX Qe FT-ICRMS instrument.

Synthesis and characterization. Synthesis and relevant characterization details are provided in the Supplementary Information.

Determination of the association constants. To quantitatively assess the inclusion complexion behavior of these compounds, fluorescence titrations of P1P5A with spm, spd, and put were performed at 298 K in a phosphate buffer solution of
Analysis of intracellular polyamines. Cells were seeded into 6-well plates at 1 × 10^5 cells/well and cultured for 24 h under 5% CO_2 at 37 °C. Then the serum-containing medium was removed, and cells were rinsed twice with PBS. Further cells were cultured with a serum-free medium containing various compounds for 6 h. After treatment, the cells were harvested by trypsinization, and then were resuspended in PBS. A part of cell suspension was used to determine the protein content according to the BCA method, and another part of the suspension was added with perchloric acid to obtain cell extract. The cell extractive was mixed with hexadeuterated and danyl chloride, and pH was adjusted to 9.5 with saturated sodium carbonate solution, reacting at 50 °C for 30 min. After cooling, ethyl acetate was added to extract the sample solution, and the concentrations of the natural polyamines (spm, spd, and put) were determined by reversed-phase high-performance liquid chromatography.

Western blotting analysis. MCF-7 cells were seeded into 6-well plates at 1 × 10^5 cells/well and cultured for 24 h under 5% CO_2 at 37 °C. Then the serum-containing medium was removed, and cells were rinsed twice with PBS. Further cells were cultured with a serum-free medium containing various compounds for 6 h at 37 °C under 5% CO_2. Following incubation, cells were washed with PBS, harvested with a scraper and centrifuged at 12 K rpm for 15 min at 4 °C. Discarding the supernatant, cells were lysed to collect the cellular protein. Equal amounts of proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% nonfat milk in Tris-buffered saline containing Tween 20 at room temperature and then incubated with rabbit anti-spermine-synthesizing antibody (1:1000), rabbit anti-spermine-synthesizing antibody (1:1000), mouse anti-ODC antibody (1:1000), and mouse anti-GAPDH antibody (1:2000), respectively, overnight at 4 °C. The PVDF membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse immunoglobulin G antibody (1:5000), respectively, for 1 h at room temperature. Immunoreactive bands were visualized with a chemiluminescence substrate kit.

In vivo antitumor efficacy. Six-week-old female Balb/C nude mice, weighing 15 g and purchased from the Beijing Experimental Animal Center (Beijing, China), were used. A total of 2 × 10^6 MCF-7 cells in 200 μL of saline were inoculated subcutaneously into the right dorsal flanks of the mice. The mice were normally fed after inoculation for 10 days. In total, 60 mice were randomly divided into 6 groups: the PBS group, free P1 group, free CP5A group, 1:1 P1 + CP5A group (all equivalent to 200 mg kg^-1 P1P5A), and two P1P5A groups (low-dose, L: 100 mg kg^-1 and high-dose, H: 200 mg kg^-1 per time). The mice were then treated through subcutaneous injection at different time points (on days 1, 3, 5, 7, and 9). The tumor volumes and the weights of the mice were recorded at different time points after treatment. The mice were sacrificed on day 11, and the tumors were separated from the animals and weighed. All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the AAALAC, and were approved by the Animal Care and Use Committee of National Beijing Center for Drug Safety Evaluation and Research. Best efforts were made to minimize the number of animals used and their suffering.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information. All data are available from the authors on reasonable request.

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Author contributions
C.L., Q.M. and J.C. conceived this project and designed the experiments. J.C. contributed to most of the experimental work. H.N., Z.M., J.W., J.X., Y.D., C.S. and Y.Z. carried out part of the experiments. C.L., Q.M., J.C., L.L. and X.J. analyzed the data. C.L., Q.M. and J.C. co-wrote the paper. All authors discussed and commented on the paper.

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