Regulation of Protein Activity and Cellular Functions Mediated by Molecularly Evolved Nucleic Acids

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Abstract: Regulation of protein activity is essential for revealing the molecular mechanisms of biological processes. DNA and RNA achieve many uniquely efficient functions, such as genetic expression and regulation. The chemical capability to synthesize artificial nucleotides can expand the chemical space of nucleic acid libraries and further increase the functional diversity of nucleic acids. Herein, a versatile method has been developed for modular expansion of the chemical space of nucleic acid libraries, thus enabling the generation of aptamers able to regulate protein activity. Specifically, an aptamer that targets integrin alpha3 was identified and this aptamer can inhibit cell adhesion and migration. Overall, this chemical-design-assisted in vitro selection approach enables the generation of functional nucleic acids for elucidating the molecular basis of biological activities and uncovering a novel basis for the rational design of new protein-inhibitor pharmaceuticals.

Proteins are central to life activities, and most biological processes involve the accurate regulation of protein activity to perform specific tasks. The exploration of molecules for protein activity modulation is important for uncovering the molecular mechanisms of biological activities,[6] disease processes,[3] and disease treatment.[3] As a class of extensively used chemical molecules with specific recognition function, a few aptamers have been identified that possess the ability to inhibit protein activity, with the advantages of strong binding affinity, high specificity, and good reversibility.[4-5] Therefore, the exploitation of aptamers to regulate protein activity is of great significance for elucidating the molecular basis of biological processes and uncovering a novel basis for the rational design of new protein-regulator pharmaceuticals.

It has been reported that the biological functions of nucleic acids are strongly coupled to their chemical space and structures.[6] Further to the canonical nucleobases, DNA/RNA molecules have various chemically diverse modifications. Over 100 naturally occurring modifications have been identified and most life activities require modified DNA/RNA to perform specific tasks.[7] Therefore, providing various modifications on nucleic acids is a promising strategy to achieve new properties and better functions. Molecularly designed artificial nucleotides could be an inexhaustible source of innovation and evolution to enrich the configurations and functions of nucleic acids for generating aptamers able to regulate protein activity.

In recent years, a series of artificial nucleotides with different functional groups have been reported.[8] These artificial nucleotides can expand the chemical space of nucleic acid libraries. Furthermore, artificially expanded libraries have been successfully employed for the selection of aptamers with high affinity and specificity to target molecules.[9] Introducing chemical groups absent in natural nucleic acids can offer new kinds of binding interactions between aptamers and their targets, such as halogen-bonding interactions and hydrophobic interactions.[10] Therefore, the use of chemical-space-expanded libraries can dramatically improve the binding affinity of aptamers, and accordingly increase the success rate of obtaining aptamers for protein activity regulation.[11]

Herein, an artificial-nucleotide-expanded (ANE) cell systematic evolution of ligands by exponential enrichment (SELEX) was developed to generate aptamers for protein-activity regulation (Scheme 1). As a proof-of-principle, nucleotides with a ferrocenyl group (Fe base), a trifluoromethyl group (F base), and a Z:P base pair was employed to demonstrate the ANE libraries. Specifically, the Fe base was selected since ferric ions can coordinate with metal-binding sites of proteins,[2] the F base was chosen because fluorine can inhibit protein activity by forming halogen bonds or amion coordination,[12] and the Z:P pair was introduced to offer a richer variety of conformations over complementary

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Moreover, the nitro moiety in Z may act as a “universal weak binder” to enhance the interactions between aptamers and proteins.\cite{9c,d,14} Using these three types of ANE libraries, ANE cell-SELEX was performed to target triple-negative breast cancer cells MDA-MB-231. The aptamer obtained by ANE cell-SELEX using the ZP-containing library was shown to inhibit the biologically relevant performance of its target, integrin alpha 3 (denoted as ITGA3). Furthermore, this aptamer can reduce ITGA3-ligand binding, thereby inhibiting tumor cell adhesion and migration. Overall, this chemical-design-assisted in vitro selection approach makes “biology-oriented synthesis” a guiding principle to harness the power of evolution in the quest for bioactive aptamers, which can, in turn, promote chemical-biology research and drug discovery.

To evolve ANE aptamers targeting MDA-MB-231 cells, ANE cell-SELEX was established (Supporting Information, Figures S1–S4 and Tables S1–S3). The aptamer of interest was chosen from the surviving library members (Supporting Information, Table S4) on the basis of clonal dominance and demonstrable binding to MDA-MB-231 cells. This aptamer was named ZAP-1 with the sequence 5’-CCCAGTGACGCAGC-CCCCGGZGGGAATTPATCGGT-GGA-CACGTTGGCTGA-3’. It was reported that the Z:P base pair is highly compatible with natural DNA, offering the six-nucleotide library a richer variety of conformations.\cite{15} As seen from the molecular structures (Figure 1a), artificial bases Z and P are structurally complementary and are similar to the natural bases in DNA, except for the nitro group in Z. Therefore, the GCATZP library would be appropriate for aptamer evolution in this situation.\cite{9d,15}

Raman spectra of the synthesized nucleic acids were measured (Figure 1b). The baseline-corrected Raman spectra of ZAP-1 (red) and natural library sample (black) are shown in the most informative spectral region, indexing to the general vibrational assignment of purine and pyrimidine residues.\cite{16} Compared to the natural library, Raman spectra of ZAP-1 (Figure 1c) show a relative increase in peak intensity at about 810 cm\(^{-1}\), which is the nitro-related feature. The existence of Z nucleobase in the synthesized oligonucleotides can be proven using Raman spectroscopy, since Z and P bases are highly similar to natural bases, except for the nitro group in Z. Confirmed by electrospray-ionization mass spectrometry (Supporting Information, Figure S5–S7) and Raman spectrometry, the above results demonstrate that Z and P bases were successfully introduced into the synthesized ZAP-1 oligonucleotides.

The specific binding capacity of ZAP-1 aptamer to target cells was determined by flow cytometry. The results (Supporting Information, Figure S8) show that ZAP-1 has obvious binding to the target cell line, but little binding to negative MCF-7 cells, suggesting that the ZAP-1 aptamer is specific to MDA-MB-231 cells. The \(K_d\) value of aptamer ZAP-1 was 48.5 nM, demonstrating that ZAP-1 aptamer can bind to MDA-MB-231 cells with high affinity (Supporting Information, Figure S9).\cite{17} These results show that ANE cell-SELEX holds great promise in developing aptamers for specific recognition.

It is known that any molecular differences between the target and control cells could lead to the selection of aptamers able to recognize such differences. Therefore, the target molecule of ZAP-1 was identified to find the unique molecular signatures expressed on the MDA-MB-231 cell surface. As shown in Figure 2a, for cells treated with proteinases, ZAP-1 exhibits a loss of its binding ability. From this, it can be deduced that the binding site of ZAP-1 was removed by the proteinases, indicating that the target molecule is most likely an extracellular membrane protein. The affinity-purified protein–aptamer complexes were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Compared with protein profiles captured by the initial library, an aptamer-specific protein band is shown in lane 5. This characteristic protein band was then digested and submitted for liquid chromatography-electrospray ionization tandem mass spectrometry identification. These results suggest that ITGA3 was the target of ZAP-1 (Supporting Information, Table S5).

To further confirm the target of ZAP-1, an aptamer pull-down experiment was performed. As shown in Figure S11 in the Supporting Information, the band for ITGA3 is clearly displayed in the aptamer-enriched sample, indicating that ITGA3 can be substantially pulled down when using aptamer
ZAP-1, but not the library. Furthermore, the binding of aptamer to target cells and to ITGA3-knockdown cells was investigated by flow cytometry. For ITGA3-knockdown cells (Supporting Information, Figures S12 and S13), ZAP-1 exhibits a loss of its binding ability, suggesting that ITGA3 suppression results in the loss of ZAP-1 aptamer binding to the target cells. As shown by confocal microscopy images (Figure 2c), green signal from ZAP-1 and red signal from anti-ITGA3 were clearly present and merged in yellow, indicating the co-localization of ZAP-1 and anti-ITGA3. (Scale bar, 50 μm.)

Figure 2. Analysis and verification of ZAP-1-targeted protein. a) Flow cytometry of fluorescein (FAM)-labeled initial library (red) and ZAP-1 (blue) with MDA-MB-231 cells treated with proteinase K (orange) and trypsin (green). b) SDS-PAGE was used to identify ZAP-1-specific band T1, marked with an asterisk. 1, marker; 2, cell lysate; 3, beads only; 4, proteins captured by initial library; 5, proteins captured by ZAP-1. c) Fluorescence confocal images of MDA-MB-231 cells incubated with ZAP-1 and anti-ITGA3. (Scale bar, 50 μm.)

It has been shown that protein-activity inhibition can be achieved by sterically blocking or altering the active domains. Much evidence indicates that integrin α3β1 displays a strong binding activity towards laminin 10 (LN10, $K_d = 7.9 \text{ nm})$. Therefore, the competitive binding ability of aptamer ZAP-1 and LN10 was investigated with flow cytometry and confocal microscopy. As shown in Figure 3a, for ZAP-1-pretreated cells incubated with LN10, the binding frequency decreased significantly, indicating a weaker affinity of LN10 to ZAP-1-pretreated cells than to untreated cells. In contrast, aptamer ZAP-1 can still effectively bind to LN10-pretreated cells. Furthermore, the binding ability of ZAP-1 and LN10 to MDA-MB-231 cells was characterized by confocal microscopy. As shown in Figure 3b, for ZAP-1-pretreated cells incubated with LN10, the green signal from ZAP-1 and the red signal from LN10 were both clearly present. Whereas, for LN10-pretreated cells incubated with ZAP-1, a strong red signal was detected, indicating a tight binding of LN10, which is not weakened by the post-treatment of ZAP-1. The quantified data of each sample measured by flow cytometry and confocal microscopy were well matched (Figure 3c,d). These results demonstrate that the binding ability of LN10 can be reduced by the pretreatment of cells with ZAP-1, and for cells pretreated with LN10, aptamer ZAP-1 can still bind to target cells.

Integrins are heterodimeric adhesion receptors for extracellular matrix proteins, consisting of an α-subunit and a β-subunit. Among these, integrin α3β1 can specifically bind to laminins, the major cell-adhesive proteins. Much evidence indicates that integrin α3β1 displays a strong binding activity towards laminin 10 (LN10, $K_d = 7.9 \text{ nm}$). Therefore, the competitive binding ability of aptamer ZAP-1 and LN10 was investigated with flow cytometry and confocal microscopy. As shown in Figure 3a, for ZAP-1-pretreated cells incubated with LN10, the binding frequency decreased significantly, indicating a weaker affinity of LN10 to ZAP-1-pretreated cells than to untreated cells. In contrast, aptamer ZAP-1 can still effectively bind to LN10-pretreated cells. Furthermore, the binding ability of ZAP-1 and LN10 to MDA-MB-231 cells was characterized by confocal microscopy. As shown in Figure 3b, for ZAP-1-pretreated cells incubated with LN10, the green signal from ZAP-1 and the red signal from LN10 were both clearly present. Whereas, for LN10-pretreated cells incubated with ZAP-1, a strong red signal was detected, indicating a tight binding of LN10, which is not weakened by the post-treatment of ZAP-1. The quantified data of each sample measured by flow cytometry and confocal microscopy were well matched (Figure 3c,d). These results demonstrate that the binding ability of LN10 can be reduced by the pretreatment of cells with ZAP-1, and for cells pretreated with LN10, aptamer ZAP-1 can still bind to target cells.

It has been shown that protein-activity inhibition can be achieved by sterically blocking or altering the active domains. The ligand-binding activity of integrins has also been reported to be dependent on their conformation and binding site availability. As demonstrated above, ZAP-1 pretreatment can decrease the amount of cells bound to
LN10 from about 80% to 40%, whereas excess LN10 pretreatment cannot block the binding of ZAP-1. Therefore, it could be possible that ZAP-1 regulates the ligand-binding activity of integrin α3β1 by sterically altering its active domains rather than blocking the binding sites. The conformation of integrin α3β1 is shown in Figure 4a, and the extracellular domains of the α3-(blue) and β1-(red) subunits are illustrated. Integrin α3β1 assumes both high-affinity (extended) and low-affinity (bent) conformers, which are in rapid equilibrium[21,23]. Previous studies have demonstrated that some molecules could regulate the binding performance of α3β1 by stabilizing one of these conformations[21,22]. In this case, it is speculated that the association of ZAP-1 with α3β1 may stabilize the low-affinity conformers (Figure 4a, right), thereby inhibiting the ligand-binding activity of integrin α3β1.[21]

The effect of ZAP-1 on MDA-MB-231 cell adhesion and migration was examined. Figure 4b shows photographs of the adhesion of MDA-MB-231 cells to LN10. After incubation, cells pretreated with ZAP-1 showed less adherence to LN10 than untreated cells. From statistical data of attached cell numbers (Figure 4c), significant inhibition of cell adhesion was observed with ZAP-1 pretreatment. These results demonstrate that ZAP-1 can inhibit MDA-MB-231 cell adhesion to LN10.

The effect of ZAP-1 on MDA-MB-231 cell migration ability was then investigated by a scratch-wound assay. After a scratch, the wound area reduced in the LN10-coated plates containing cells, whereas the cells still had a wide gap in the ZAP-1-treated LN10 precoated plate (Figure 4d). The closure degree of the scratch area was expressed as percentage of the initial scratch area to quantify cell migration. Importantly, ZAP-1 pretreated cells significantly delayed wound closure compared to cells treated with initial library (Figure 4e). All the above results suggest that ZAP-1 can markedly reduce cell adhesion and migration ability by regulating integrin α3β1 activity, which stands in good agreement with the speculation mentioned before. Therefore, aptamer ZAP-1 could be a potential inhibitor of tumor metastasis.

In this work, we have established a versatile strategy for generating functional nucleic acids by introducing artificial nucleotides with specific chemical groups to the SELEX process. With this strategy, the ZAP-1 aptamer, which can bind to and further regulate the activity of the target protein, was developed. Once bound, it inhibits integrin α3β1-mediated adhesion and migration of MDA-MB-231 cells. Moreover, the aptamer obtained here shows potential for inhibiting metastatic activity in preclinical research. This study serves as a model for aptamer mediation of cellular and molecular interactions and demonstrates the capabilities of aptamers as protein-activity regulators. In conclusion, this chemical-design-assisted in vitro selection approach makes “biology-oriented synthesis” a guiding principle with which to harness the power of evolution in the quest for bioactive aptamers.

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Figure 4. Modulation of integrin α3β1 by ZAP-1 combination. a) Model for conformational modulation of integrin α3β1 by ZAP-1 binding. b) MDA-MB-231 cell adhesion result. (Scale bar, 200 μm.) c) Attached cell numbers in three separate fields expressed as percentages of the cell number after LN10 incubation. d) Cell migration result. e) Closure degree of the scratched area was expressed as percentage of the initial scratched area. Each bar represents the mean ± SD.
Conflict of interest

The authors declare no conflict of interest.

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