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SS30, a novel thioaptamer targeting CD123, inhibits the growth of acute myeloid leukemia cells

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A R T I C L E I N F O

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A B S T R A C T

Aims: CD123 represents an important acute myeloid leukemia (AML) therapeutic target. CD123 aptamers may potentially serve as tumor-homing ligands with excellent affinity and specificity for AML targeted therapy, but their complexity, laborious preparation and nuclease digestion limited pharmacological application. The aim of this study was to develop the first CD123 thioaptamer to overcome these obstacles.
Main methods: Flow cytometry was utilized to assess the binding specificity, affinity and anti-nuclease ability of thioaptamer. CCK8, Annexin-V/DAPI, and colony forming assays were used to evaluate the anti-cancer ability of thioaptamer *in vitro*. The tumor volume, weights, survival rate, H&E staining of organs, and serum level of organ damage biomarkers of animal model were applied to investigate the anti-cancer ability of thioaptamer *in vivo*. Furthermore, we explored the binding mechanism between thioaptamer and CD123.
Key findings: CD123 thioaptamer SS30 was able to bind to CD123 structure with high specificity in complex nuclease environment, the dissociation constant of 39.1 nM for CD123 peptide and 287.6 nM for CD123$^+$ AML cells, while exhibiting minimal cross-reactivity to albumin. Furthermore, SS30 inhibited the proliferation and survival of AML cell lines and human AML blasts selectively *in vitro* ($P < 0.01$). In addition, SS30 prolonged the survival and inhibited tumor growth in a mouse xenograft tumor model *in vivo*. Of note, SS30 blocked the interaction between IL-3 and CD123, and decreased expression of p-STAT5 and p-AKT.
Significance: The proliferation inhibition and nuclease resistance ability of SS30 made it as a more promising functional molecule for AML targeted therapy.

1. Introduction

Acute myeloid leukemia (AML) is one of the most common types of leukemia in China. AML features a differentiation arrest and uncontrolled, abnormal clonal proliferation of myeloid precursors, resulting in impairments of hematopoiesis [1]. AML has diverse outcomes and remains one of the most complex and challenging diseases in contemporary oncology [2]. After decades of stagnation in the armamentarium for AML, chemotherapy remains the foremost therapeutic option for AML [3]. However, one of the major issues of chemotherapy is its adverse effects, which limit the effect of chemotherapy [4]. At present, targeted therapy is one of the most effective methods for treating AML. Antigens expressed specifically on AML cells or those whose expression on normal cells are much lower have become ideal targets for AML targeted therapy. CD123, the α-subunit of the interleukin-3 receptor (IL-3Rα), may form a heterodimer with the signal transducing β chain, and mediate the proliferation, differentiation and apoptosis of AML cells *via* AKT and JAK-STAT5 signal pathways [5,6]. CD123 expression is not only upregulated on AML blasts with high levels reported to be associated with aggressive disease, but is also low or not present on primitive hematopoietic stem cells, erythroid progenitor cells, mature granulocytes and lymphocytes [7]. Furthermore, high

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numbers of $CD34^+CD38^{low/-}CD123^+$ blasts predict poor outcome in AML patients and the $CD34^+CD38^-CD123^+$ population is increased in AML patients with a relapse [8]. Therefore, CD123-directed AML therapies may have the ability to debulk and eliminate AML the furthest, potentially translating to more durable therapeutic responses [9,10]. At present, CD123 antibodies remain the major targeting agents for AML [11,12]. CD123 antibodies applied in the clinical treatment of AML, including Antibody-Drug Conjugate (ADC) [13], Bispecific T-cell engager (BiTE) [14] and Chimeric Antigen Receptor T-Cell Immunotherapy (CART) [15], have exhibited a marked targeting ability, excellent tolerance and favorable safety, indicating that CD123-targeting therapeutics are promising treatments [16]. However, the antibodies caused capillary leak syndrome as well as relatively frequent hepatic transaminase elevation, hypoalbuminemia and myelosuppression [17]. Thus, novel CD123-targeting therapeutics with improved features over those of the approved existing methods in AML treatment are urgently required.

Aptamers are a class of molecular targeting molecules composed of single-stranded oligonucleotides (DNA or RNA) and may form a specific three-dimensional (3D) structure [18]. Aptamers may recognize their targets with high specificity and affinity [19]. When compared with antibodies, aptamers have the advantages of easy synthesis, chemical modification, limited synthesis cost and small size, allowing them to penetrate solid tumors, and no immunogenic properties, which may facilitate long-term therapeutic efficacy and safety [20]. Aptamer-mediated targeting systems have been demonstrated to have great potential for AML therapy [21,22]. They are typically constructed by linking aptamers with drug-loading complexes, including liposomes [23], micelles [24], nanoparticles [25] or dendrimers [26]. However, aptamer-drug complexes possess specific limitations: Sophisticated design, laborious preparation and limited drug loading [27]. These obstacles represent a bottleneck for the production scale-up. Furthermore, due to their poor biodegradability, the drug-loading particles exhibit chronic organ accumulation and produce further inflammation *in vivo* [28]. Therefore, to circumvent these problems, novel AML therapeutics based on CD123 aptamers are urgently required.

Since the first RNA aptamer was developed in 1992 [29], triggering the rapid development of further aptamers, studies have indicated that aptamers not only bind to their targets with high specificity and affinity, but also inhibit the targets' biological activities. After the approval of the first aptamer drug Macugen by the Food and Drug Administration for the treatment of age-associated macular degeneration, an increased amount of aptamers that are able to inhibit their targets' biological functions have been developed [30]. Although the concept of bio-inhibition by aptamers has been developed for < 20 years, marked progress in laboratory experiments and clinical trials has been made. When compared with traditional aptamer-mediated targeting systems, these bio-inhibition aptamers capable of inhibiting tumor cells were lacking a sophisticated design and laborious preparation [31,32]. However, to the best of our knowledge, no previous study has reported on CD123 aptamers with the ability to inhibit $CD123^+$ AML tumor cells.

The present study reports on SS30, a novel therapeutic aptamer targeting AML, the first ever thioaptamer, which was derived from CY30, a previous CD123 aptamer reported by our group [22]. It was demonstrated that SS30 is able to bind CD123 with high specificity and affinity. SS30 was more resistant to nuclease digestion when compared with regular aptamers. In addition, SS30 had a marked inhibitory effect on $CD123^+$ AML tumor cells *in vitro* and *in vivo*. Furthermore, SS30 produced a reduced amount of adverse effects and prolonged survival of model animals *in vivo*. Finally, it was demonstrated that SS30 impaired the function of CD123 molecule by competing with IL-3 to bind to CD123, and could down-regulate the expression of p-AKT and p-STAT5. Overall, the present *in vitro* and *in vitro* data as well as mechanistic studies fully validate that SS30 is a novel anti-tumor agent with therapeutic potential for AML.

2. Materials and methods

2.1. Reagents

The single-stranded (ss) DNA library and oligonucleotide primers were synthesized by Sangon Biotech (Shanghai, China). CD123 peptides with at least 95% purity were synthesized by SBS Genetech (Beijing, China). Bovine serum albumin (BSA) was obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany; cat. no. V900933) and (*S*p)-20-deoxyadenosine-50-*O*-(1-thiotriphosphate) [dATP(αS)] was purchased from GE Healthcare (Little Chalfont, UK). A Cell Counting Kit 8 (CCK-8) was obtained from Sigma-Aldrich (Merck KGaA; cat. no. 96992) was from Beyotime Institute of Biotechnology (Haimen, China). Annexin-V-APC (cat.no.550475), phycoerythrin-conjugated anti-CD123 antibody (cat. no. 555749), and p-STAT5-Alexa Fluor 647 (cat.no.612599) were purchased from BD Biosciences (San Jose, CA). p-AKT-Alexa Fluor 647 (cat.no.4075, Beverly, MA) was purchased from Cell Signaling Technology (USA). FITC-conjugated anti-HER2 antibody (ThermoFisher, USA; BMS120FI). Mono-dispersed magnetic urea–formaldehyde microspheres were purchased from Baseline Chromtech (Tianjin China).

2.2. Patient samples and analysis

The protocol of the study using patient samples was reviewed and approved by the Ethics Committee of Xi'an Jiaotong University Affiliated Children's Hospital (Xi'an Children's Hospital, Xi'an, China). 50 AML patients (7.18 ± 2.97 yrs) at diagnosis or at relapse from January 2015 to December 2017 agreed to sign informed consent. Bone marrow (BM) or peripheral blood (PB) samples were obtained, and information on the patients is provided in Supplemental Table S1. Patients were classified by laboratory and genetics examinations, based on the National Cancer Center Network criteria [33]. The cytogenetics of patients who were with −5, 5q-, −7, 7q-,11q23 other than t(9;11), inv(3;3), t(6;9), t(9;22), complex findings(≥3 clonal chromosomal abnormalities), or FLT3-ITD mutated were divided into high risk group; The cytogenetics of patients who were with normal cytogenetics, +8, t(9;11), or other chromosomal abnormalities were divided into mediate risk group; The cytogenetics of patients who were with Inv(16), t(16;16), t(8;21), normal cytogenetics with NPM1 mutation or isolated CEBPA mutation in the absence of FLT3-ITD were divided into low risk group(Supplemental Table S2). Mononuclear cells (MNCs) were isolated by Ficoll-Hypaque (Sigma Diagnostics; Livonia, MI, USA) separation. The expression of CD123 and human epidermal growth factor receptor-2 (HER2) on AML blasts from patients were evaluated using phycoerythrin-conjugated mouse anti-CD123 antibody (BD Biosciences, San Jose, CA, USA; cat. no. 555749) and FITC-conjugated anti-HER2 antibody (ThermoFisher, USA; BMS120FI), respectively. HER2 was treated as negative bio-marker, Cells were washed by PBS and incubated with anti-CD123 antibody at 37 °C for 30 min. Cells were washed by PBS and analyzed by flow cytometry. The mean fluorescent values were calculated.

2.3. Cell lines and culture

The Molm-13 human acute myelocytic leukemia, HNT34 human myeloid leukemia and RCH-ACV human B-cell precursor leukemia cell lines were obtained from Cell Culture Centre of Peking Union Medical College (Beijing, China) in 2016. The human AML KG-1a [American Type Culture Collection (ATCC) no. ATCC-CCL-246.1™], acute monocytic leukemia THP-1 (no. ATCC-TIB-202™), Biphenotypic B myelomonocytic leukemia MV4-11 (no. ATCC-CRL-9591™), acute promyelocytic leukemia HL60 (no. ATCC-CCL-240™) and erythroleukemia HEL-92.1.7 (no. ATCC-TIB-180™) cell lines were purchased from ATCC (Manassas, VA, USA). All of these cell lines and the patients' primary AML blasts were cultured in RPMI-1640 medium supplemented with

10% fetal bovine serum (FBS, Gibco) and a mixture of penicillin/streptomycin. Cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2. All experiments were performed on cells in the exponential growth phase. The expressions of CD123 and HER2 on these cell lines were assessed. HER2 was treated as the negative bio-marker.

2.4. Preparation of CD123 thioaptamer CY30

To generate thioaptamer, CY30 was thiophosphorylated by 5′-monothiophosphate substitutions of all dA. CY30 was amplified by polymerase chain reaction (PCR; 30 cycles of 30 s at 94 °C, 30 s at 60 °C and 1 min at 72 °C, followed by 10 min at 72 °C) in a reaction system containing dATP(αS)(20 mM), dCTP (20 mM), dGTP (20 mM), dTTP (20 mM), $MgCl_2$ (2 mM), fluorescein isothiocyanate (FITC)-primer 1 (5′-TGCGTGTGTAGTGTGTCTG-3′) or biotin-primer 2(5′-CCGCCCAAAT CCCTAAGAG-3′), and Taq polymerase (0.5 U, Sangon Biotech, Shanghai, China). After the amplification, thionylated CY30, which was termed as SS30, was separated by using streptavidin-coated magnetic beads (Promega Corp., Madison, WI, USA).

2.5. In vitro binding assays

To evaluate the binding specificity of SS30 to CD123 peptide, FITC-labeled SS30 was incubated with CD123- and BSA-coated magnetic beads (1×10^6/mL) in 37 °C for 30 min, respectively. The sequence of CD123 peptide is: TDIE<u>C</u>VKDADYSMPAVNNSY<u>C</u>QFG. This sequence is a part of CD123 extracellular domain predicted from Protein Data Bank (PDB). We modified a disulfide bond between two cysteines which were underlined to simulate its natural structure maximally. Subsequently, the beads were washed by PBS twice and analyzed by flow cytometry. Thio-random aptamer was applied in the control group. To then assess whether SS30 was able to recognize CD123$^+$ cells selectively, FITC-labeled SS30 (10 mM) was incubated with either 1×10^5 Molm-13, KG-1a, RCH-ACV or EL4 cells at 37 °C for 30 min. The cells were analyzed by flow cytometry. To determine the binding affinity of SS30 to the structure of CD123, CD123- or BSA-coated magnetic beads were incubated with gradient concentrations of FITC-labeled aptamer at 37 °C for 30 min. The beads were washed three times, re-suspended in 0.2 ml PBS and subjected to flow cytometric analysis. The binding assay was repeated three times. The mean fluorescence intensity of target labeled by aptamers was used to quantify specific binding by subtracting the mean fluorescence intensity of non-specific binding to BSA-coated beads. The equilibrium dissociation constants (Kd) of the aptamer-CD123 interaction were obtained by fitting the dependence of fluorescence intensity of specific binding on the concentration of the aptamers to the equation $Y = B_{max} X / (Kd + X)$, where Y represented the reciprocal of the average fluorescence intensity, X represented the reciprocal of thioaptamer's concentration, and Bmax represented the maximum binding capacity of aptamer bound to CD123 peptide. Furthermore, to assess the SS30 binding affinity to CD123$^+$ AML cells, FITC-labeled SS30 (10 mM) was incubated with either 1×10^5 Molm-13 or KG-1a cells at 37 °C for 30 min. The cells were analyzed by flow cytometry. The binding affinities to Molm-13 and KG-1a were calculated as mentioned above.

2.6. Identification of SS30's target

Since there are many kinds of proteins expressed on cells, To further demonstrate the binding target of SS30 was CD123, 1×10^5 Molm-13 cells were incubated with PE-conjugated anti-CD123 antibody and various concentrations of FITC labeled SS30 (10, 50, 100, 150, and 200 μM) at 37 °C for 30 min. Then, cells were washed by PBS twice and analyzed by flow cytometry. The mean fluorescence intensities (MFI) of CD123 antibody and SS30 were analyzed.

2.7. Nuclease resistance assay of SS30

To evaluate the bio-stability of SS30 and to assess whether it was still able to function in presence of nuclease, SS30 and CY30 were incubated with human serum for different durations (0–168 h). Samples were then subjected to 1% agarose gel electrophoresis or added to CD123$^+$ cells and incubated for 30 min. The band density was analyzed using gene tools software from Syngene. Cells were washed by PBS twice and analyzed by flow cytometry.

2.8. In vitro cytotoxicity assay

Cell lines or AML blasts from patients were incubated and collected, washed with PBS and seeded in a 96-well plate (5×10^4 cells per well) in absence or presence of IL-3. Cells were treated with SS30 (20 mM), CY30 (20 mM), PBS, or thio-random library(5′-TGCGTGTGTAGTGTGT CTG-(N28)-CTCTTAGGG.

ATTTGGGCGG-3′, 20 mM) or for 1 h. The cells were then washed with PBS and cultured for a further 36 h. Subsequently, a CCK-8 assay was used to determine cell viability according to the manufacturer's standard protocol.

2.9. Colony-forming assays

CD34$^+$ cells from patients were plated in methylcellulose progenitor culture and dispensed into 35-mm dishes, which was suspended in 0.3 ml Iscove's modified Dulbecco's medium (HyClone) with 30% FBS, 3 U/ml erythropoietin, 5 ng/ml IL-3, 5 ng/ml stem cell factor, 20 ng/ml granulocyte colony-stimulating factor and 20 ng/ml granulocyte-macrophage colony-stimulating factor. Cells were treated with SS30 (20 mM), CY30 (20 mM), thio-random library(5′-TGCGTGTGTA GTGTGTCTG-(N28)-CTCTTAGGGATTTGGGCGG-3′, 20 mM) or PBS. After 14 days, hematopoietic colonies were counted.

2.10. Apoptosis and cell death analysis of AML cell lines and AML blasts from patients

CD123$^+$ AML cell line Molm-13 and CD123$^-$ cell line RCH-ACV were seeded in 6-well plates. Cells were incubated with SS30 (10, 50, 100, and 200 nM), or PBS for 2 h. Then, to assess the apoptosis of cells caused by SS30, cells were incubated with Annexin-V-APC and 4′6-diamidino-2-phenylindole (DAPI). Cells were detected by flow cytometry and analyzed by using Flowjo software (Tree Star, Ashland, OR). Further, to observe cell death visibly, the cells were stained by Hoechst/PI dye. Cells were analyzed by laser scanning confocal microscope. For the apoptosis of AML blasts from patients, AML blasts were incubated and collected, washed with PBS and seeded in 96-well plates (5×10^4 cells per well). Cells were treated with SS30 (5,10, 15, 20, 25, 50, 100, 150, 200, 250, 400 and 500 nM), thio-random library (5,10, 15, 20, 25, 50, 100, 150, 200, 250, 400 and 500 nM) or PBS for 1 h. Subsequently, the cells were washed with PBS and cultured for a further 36 h. The apoptosis of cells were analyzed by standard protocols outlined by the manufacturer.

2.11. Evaluation of anti-cancer effects of SS30 in vivo

The protocol of the animal study in this paper was reviewed and approved by the Ethics Committee of Xi'an Jiaotong University Affiliated Children's Hospital (Xi'an Children's Hospital, Xi'an, China), no. C2018004. 8-week-old female BALB/c mice were purchased from the Xi'an JiaoTong University Lab Animal Centre (Xi'an) and raised under pathogen-free conditions. Firstly, to assess whether SS30 could recognize murine CD123 as well, murine CD123 protein was coated on magnetic beads, and incubated with FITC-labeled SS30 or random DNA at 37 °C for 30 min. Beads were washed by PBS twice and analyzed by flow cytometry.

Secondly, to evaluate the anti-cancer effect of SS30, the mouse xenograft tumor model was generated by subcutaneously injecting 2×10^7 *in vitro*-propagated Molm-13 cells into the flank of BALB/c mice. Dorsal tumor nodules were allowed to grow to a volume of $\sim 100\,mm^3$ prior to treatment initiation. Tumor-bearing mice were randomly divided into three groups, with six in each group: i) treated with SS30 (2 mg/kg); ii) treated with thio-random library (2 mg/kg); and iii) treated with saline. Drugs were administered through tail vein injection once a day. The tumor volume was calculated using the following equation:

$$\text{Tumor volume} = \text{Tumor Length} \times \text{Tumor Width}^2/2.$$

To evaluate whether SS30 may cause any adverse effects or cause damage to normal tissues, whole blood was collected and evaluated for serum markers of organ damage [creatine kinase muscle/brain (CK-MB), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and blood urea nitrogen (BUN)] and serum-associated inflammatory cytokines [tumor necrosis factor (TNF)-α and interferon (INF)-α]. Subsequently, when the tumor volume was over $6000\,mm^3$, or the mice that lost over 15% of their pre-treatment body weights, the mice were euthanized by broken neck. The comprehensively judgment of death was by the observation of respiratory, heartbeat, pupil, and nerve reflex. After the confirmation of death, the heart, liver, kidney, spleen, stomach and lung were collected and subjected to hematoxylin and eosin (H&E) staining to evaluate any adverse effects of SS30 on normal tissues.

2.12. Molecular docking

The protein-protein module of MOE is applied for protein docking with the adaptor. Before molecular docking, we selected 5UWC (PDB ID) as the docking receptor protein of CD123. The resolution of 2.40 Å, the sequence interval is 20–307. The SS30 aptamer was constructed by MOE construction module, and the energy was minimized. The docking parameters are set as rigid docking. The Pymol was used to analyze hydrogen bond interactions in the docking position.

2.13. Competition assay

1×10^5 cells (KG-1a, Molm-13, HNT-34, THP-1, MV4-11 or HL60) were incubated at 96-well plate and simultaneously with PBS, 50 nM IL-3 and increasing concentrations of FITC-labeled SS30 and random library, ranging from 50 to 100 nM for monovalent aptamers, for 1 h at 37 °C. The cells were washed with PBS and incubated for a further 48 h. Cell culture supernatant fluids were collected at 0, 10, 20, 30 and 40 h, and were analyzed with a CCK-8 kit to determine the cell viability.

2.14. Assessment of p-AKT and p-STAT5 expression

To evaluate whether SS30 could cause dynamics change of signal transduction pathway, Molm-13 cells were incubated with SS30 (10, 50, 100, and 200 nM), random DNA (200 nM), or PBS for 2 h. Cells were cultured for another 12 h and fixed with paraformaldehyde at a final concentration of 1.6% at room temperature for 10 min. Cells were washed and stained with p-STAT5-Alexa Fluor 647 or p-AKT-Alexa Fluor 647 for 30 min at room temperature in darkness. After being washed twice, cells were analyzed by flow Cytometer and MFI values were recorded.

2.15. Statistical analysis

SPSS11.0 software was applied from Xi'an Jiaotong University. All numerical data were expressed as the mean ± standard deviation. Differences between the groups were examined with Student's two-tailed *t*-test or one-way ANOVA using SPSS11.0 software from Xi'an Jiaotong University. ANOVA was performed to evaluate the difference followed by Tukey *post hoc* test. Kaplan Meier analysis was used to analyze the overall survival. Independent sample t-test was used to analyze the variance of experimental design. *P*-values of < 0.05 were considered statistically significant. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

3. Results

3.1. CD123 is overexpressed on AML blasts from patients

It has been indicated that CD123 is specifically overexpressed on AML blasts [11]. To further confirm the expression levels of CD123, the AML blasts of 50 patients were examined using flow cytometry. As presented in Supplemental Table S1, various morphological subtypes and AML samples with high-risk molecular genetic abnormalities were included. In line with previous studies, CD123 was detected in the samples of all 50 (100%) patients. HER2 was also detected as a negative control. As shown in Supplemental Table S1, when compared with HER2 expression, these 50 patients had a relative high CD123 expression on their AML blasts, the mean fluorescence intensity is 2186.96 ± 169.6, which is also higher than that in the normal people samples [34]. The expressions of HER2 were relatively low. Again, these data suggest the potential application of CD123 for AML targeted therapy.

3.2. Generation of SS30

In a previous study by our group, CD123 aptamer CY30 was selected by using the SELEX technique. CY30 was confirmed as an ideal target molecule that may bind to the structure of CD123 with high specificity and affinity. To enhance the nuclease binding ability of CY30, PCR was used to change the A base of CY30 into a monothiophosphate-modified A base. The PCR product was analyzed by 1.0% agarose gel electrophoresis. As presented in Fig. 1A, the length of SS30 was 66 bp. The sequence of SS30 is: 5′-TGCGTGTGTACTGTGTCTGGTCCCGTAGCTAC TAGCGAACTCCCTGCCTCTTAGGGATTTGGGCGG-3′. The second structure of SS30 was predicted by M-fold software and presented in Supplemental Fig. 1.

3.3. Binding properties of SS30

To evaluate whether SS30 retained its binding specificity to the structure of CD123, binding specificity tests were performed. BSA was used as a negative control, as it is abundant in blood. In general, FITC-labeled SS30 or random DNA were incubated with BSA or CD123 peptide-coated beads and analyzed by flow cytometry. As presented in Fig. 1B, SS30 exhibited a high affinity to CD123 and a relatively weak binding to BSA. These results indicated that SS30 was able to recognize CD123 selectively.

Although SS30 was demonstrated to bind to CD123 peptide, it remained elusive whether SS30 could bind to $CD123^+$ cells selectively. To address this question, firstly, the CD123 expressions on $CD123^+$ (Molm-13, KG-1a, THP-1, MV4-11) and $CD123^-$ (RCH-ACV, HNT-34, HL-60, and HEL-92.1.7) cells were detected. HER2 was treated as negative markers. As shown in Supplementary Fig. 2, all $CD123^+$ cells expressed a relatively high expression of CD123 and low expression of HER2, $CD123^-$ cells expressed both low CD123 and HER2. These results were in accordance with previous reports. Then, FITC-labeled SS30 was incubated with either $CD123^+$ cells (Molm-13 and KG-1a) or $CD123^-$ cells (RCH-ACV and EL4) under the same experimental conditions and the cells were analyzed by flow cytometry. FITC-labeled random DNA was used as a background control. As presented in Fig. 1C, SS30 exhibited strong binding to Molm-13 and KG-1a, whereas binding to RCH-ACV and EL4 was weak. Furthermore, random DNA exhibited no binding preference to any of the cell lines. These results indicated that SS30 could bind to $CD123^+$ cells selectively.

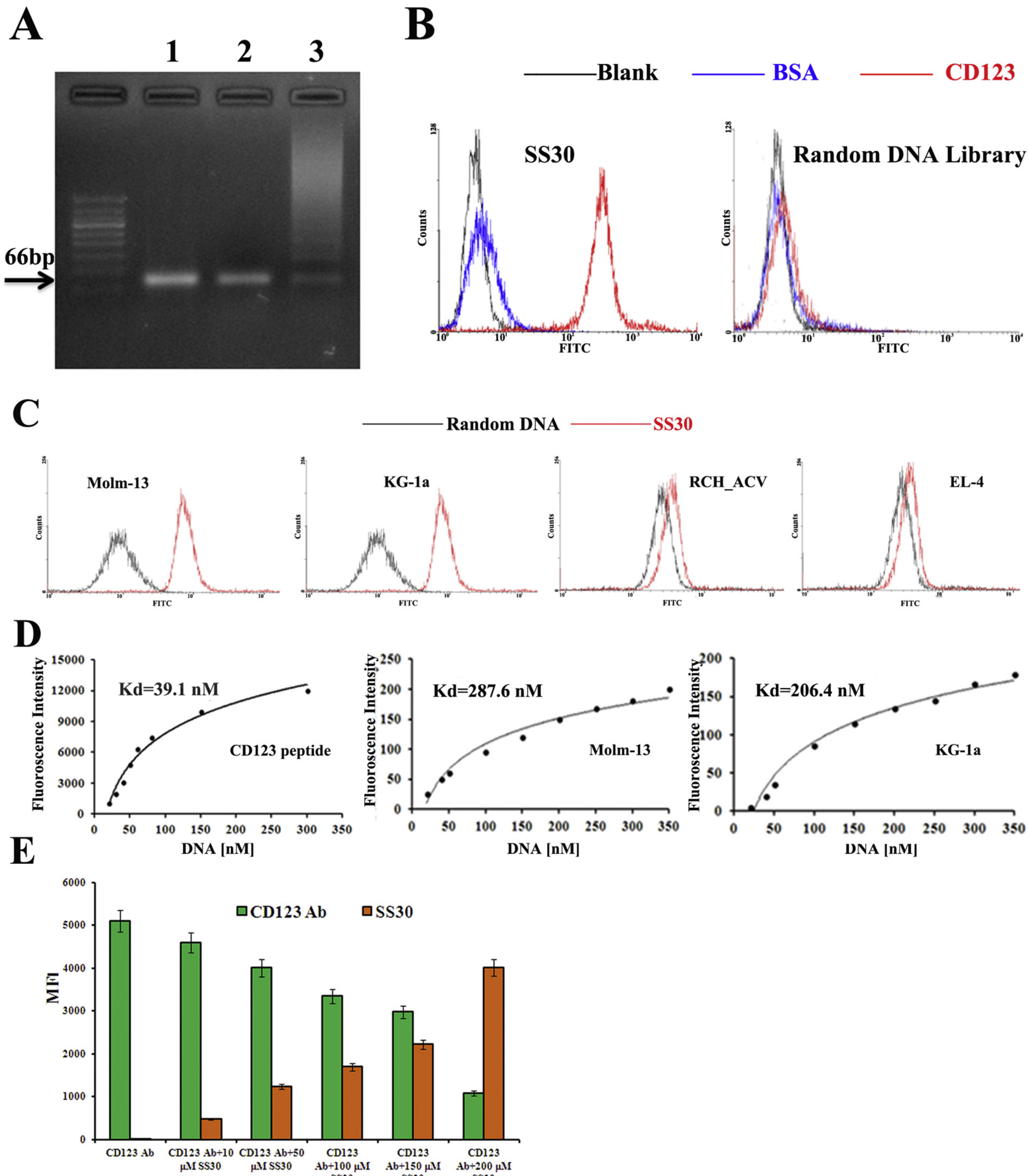

Fig. 1. Characterization of thioaptamer SS30. (A) Assessment of the length of SS30. Random DNA library amplified in normal PCR system (lane 1), CY30 amplified in normal PCR system (lane 2), and CY30 amplified in mono-thiophosphate modified A base PCR buffer (lane 3). (B) Flow cytometric evaluation of the binding specificity to CD123 peptide or other negative proteins by FITC-labeled thioaptamer SS30. The black curves represent the control fluorescent signals generated by FITC-labeled random DNA from the library pool. (C) Flow cytometric assessment of the binding specificity of SS30 to $CD123^+$ and $CD123^-$ cells. The black curves represent the control fluorescence signals generated by FITC-labeled random DNA from the library pool. (D) Flow cytometric evaluation of the binding affinities to CD123 peptide, $CD123^+$ AML cell line Molm-13, and KG-1a by FITC-labeled aptamers SS30. (E) SS30 compete CD123 antibody to bind with CD123 structure.

Furthermore, to evaluate the binding affinity of SS30, various concentrations of FITC-labeled SS30 were incubated with CD123 peptide-coated magnetic beads or $CD123^+$ AML cells at 37 °C for 30 min. The beads or cells were washed with PBS twice and analyzed by flow cytometry. FITC-labeled random DNA was applied as a background control. The experiments were repeated three times. Non-linear regression analysis revealed that SS30 had a Kd of 39.1 nM for CD123 peptide, a Kd of 287.6 nM for Molm-13 cells, and a Kd of 206.4 nM for KG-1a cells (Fig. 1D).These results indicated that SS30 preferentially recognizes and binds with $CD123^+$ cells *in vitro*.

3.4. SS30 recognizes CD123 on AML cells

SS30 could bind to $CD123^+$ cells with high specificity and affinity, however, there were many kinds of proteins expressed on AML cells. Thus, there was a need to explore whether the target of SS30 was

CD123. Molm-13 cells were incubated anti-CD123 antibody and various concentrations of FITC labeled SS30. The mean fluorescence intensities (MFI) of CD123 antibody and SS30 were analyzed respectively. As presented in Fig. 1E, with SS30 concentration was increasing, the MFI of CD123 antibody decreased significantly, and the MFI of SS30 increased. Thus, SS30 could compete with CD123 antibody to bind with CD123, which indicated that CD123 may be the target of SS30.

3.5. Biostability of SS30 against nucleases

To test the anti-nuclease ability of SS30 and whether it was still able to function in the presence of nucleases, SS30 and CY30 were incubated with human serum for different durations (0, 6, 12, 24 or 48 h). Samples were subjected to 1% agarose gel electrophoresis or added to Molm-13 for 30 min. As presented in Fig. 2A, SS30 maintained integrity in the presence of serum when compared with CY30. As indicated in Fig. 2B, after the incubation with serum, only SS30 exhibited a strong binding trend to Molm-13, whereas CY30 was unable to undergo such binding. These results demonstrated that the thiophosphorylated SS30 was more resistant to nuclease than unmodified CY30, and may retain its efficacy in a complex internal environment. When compared with CY30 and SS30, the physical properties of them were quite similar except for anti-nuclease resistance (Supplemental Table S3).

3.6. SS30 inhibits the proliferation of on CD123^{+} cell lines in vitro

To evaluate the potential anti-proliferation effect of aptamer SS30 on CD123^{+} cells, the cell lines Molm-13, KG-1a, THP-1 and HNT34 (CD123^{+}), and RCH-ACV, MV4-11, HL60 and HEL-92.1.7 (CD123^{-}) were treated with SS30, thio-random library or PBS for 1 h. Subsequently, the cell viabilities of cell lines were determined using CCK-8 assay. As presented in Fig. 3A, when compared with cells incubated with IL-3, cell viabilities in absence of IL-3 were lower, indicating that IL-3 could stimulate the proliferation of CD123^{+} AML cells. In addition, when compared with thio-random library and PBS, SS30 significantly inhibited the proliferation of the four CD123^{+} cell lines ($P < 0.01$). In CD123^{-} cell lines (HL60, RCH-ACV. MV4-11, and EL-4), IL-3 did not cause significant difference between in the presence or absence of IL-3, and SS30 did not affect the cell viabilities obviously. Furthermore, cell apoptosis was assess by Annexin-V/DAPI. As presented in Fig. 3B, with the increasing of SS30 concentration and incubation time, the apoptosis rate of Molm-13 cells increased significantly ($P < 0.01$), whereas apoptosis rate of RCH-ACV did not change obviously. In addition, to further observe cell death more visually, confocal microscope was applied. As presented in Fig. 3C, when compared with PBS and random DNA group, cell death rate of CD123^{+} cell Molm-13 treated with SS30 was much higher, whereas there were obvious difference in CD123^{-} cell RCH-ACV cell treated with either SS30, random DNA, or PBS. As expected, SS30 significantly induced apoptosis of Molm-13 cell lines when compared with thio-random library and PBS. Therefore, these data indicated that SS30 inhibits the proliferation and induces apoptosis of CD123^{+} AML cells.

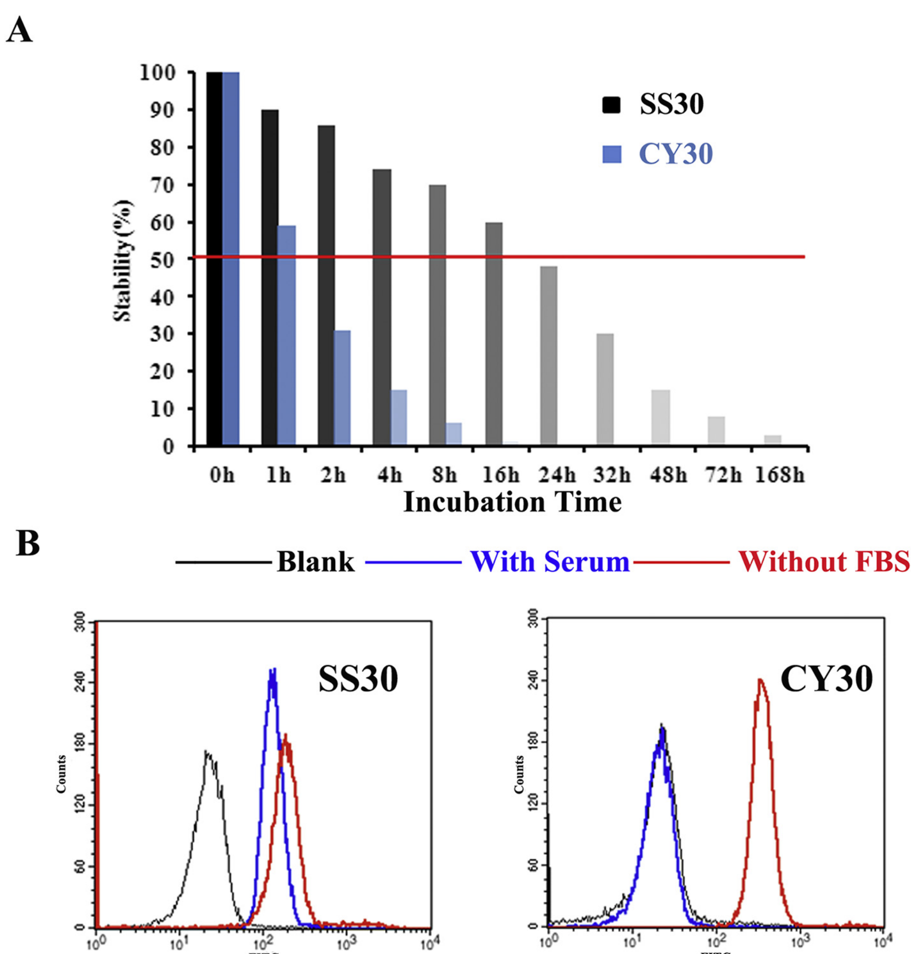

Fig. 2. Nuclease resistance evaluation. (A) Agarose gel electrophoresis to confirm DNA integrity after incubation with human serum at 37 °C for different durations. (B) Flow cytometric analysis of the binding specificity of SS30. FITC-labeled SS30 and CY30 were incubated with human serum for different durations (0–168 h). Samples were added to CD123^{+} cells and incubated for 30 min. Cells were washed by PBS twice and analyzed by flow cytometry.

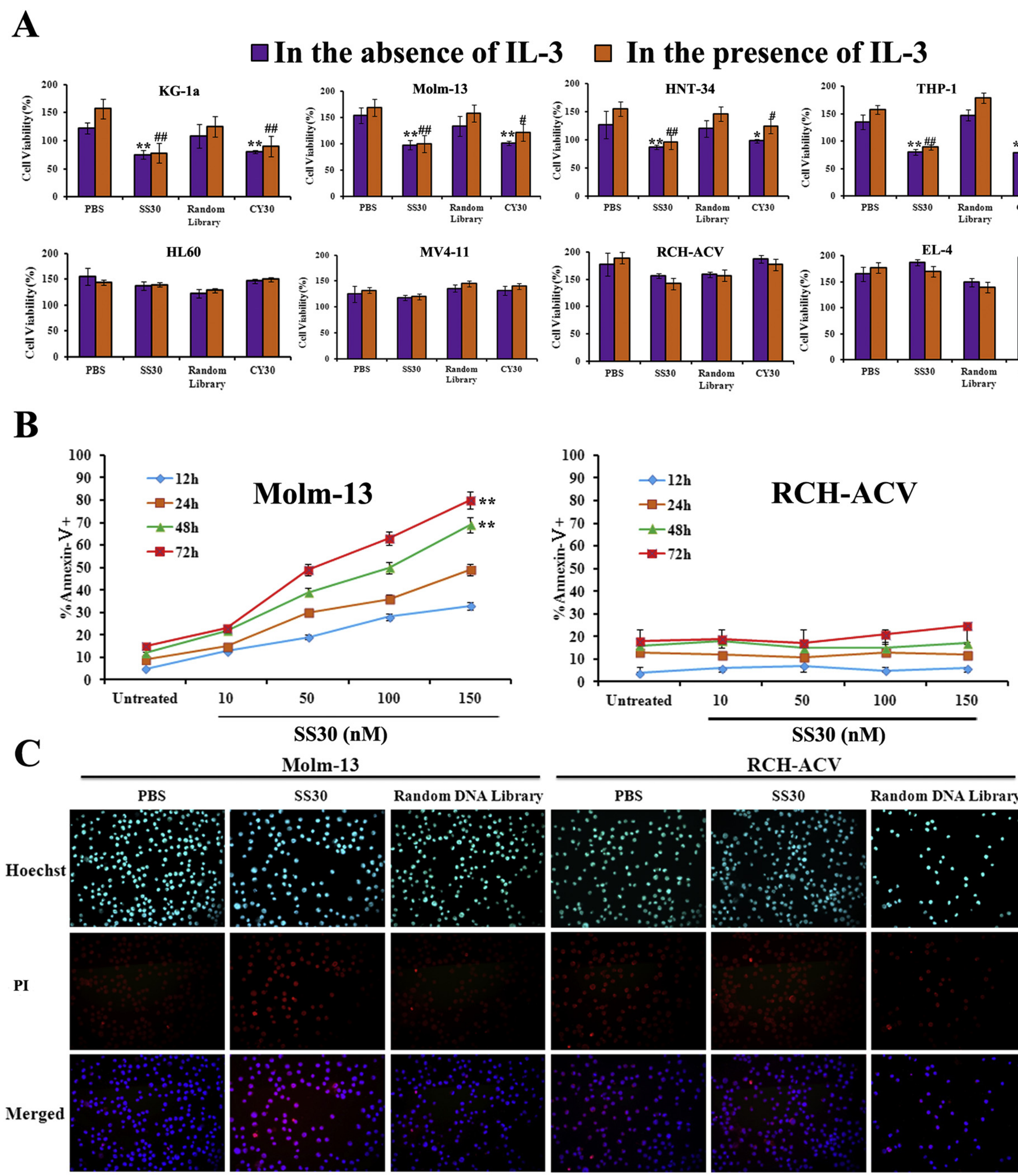

Fig. 3. SS30 inhibits the proliferation and induces cell apoptosis and death of CD123$^+$ cells *in vitro*. (A) Cell Counting Kit-8 assessment of cell proliferation. Cell lines were treated with SS30, CY30, thio-random library or PBS for 1 h. The cells were then washed with PBS and cultured for a further 36 h. CCK-8 assay was used to determine cell viability. $^{**}P < 0.01$, $^{*}P < 0.05$ (Compared with cell treated with PBS in absence of IL-3); $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ (Compared with cell treated with PBS in presence of IL-3). (B) Analysis of cell apoptosis. CD123$^+$ AML cell line Molm-13 and CD123$^-$ cell line RCH-ACV were incubated with SS30, random DNA or PBS for 2 h. Cells were washed with PBS and stained by Annexin-V/DAPI dye. Cells were analyzed by flow cytometry. (C) Analysis of cell death. Molm-13 and RCH-ACV were incubated with SS30, random DNA or PBS for 2 h. Subsequently, the cells were washed with PBS and stained by Hoechst/PI dye. Cells were analyzed by laser scanning confocal microscope.

3.7. *SS30 inhibits the proliferation and survival of AML blasts from patient specimens in vitro*

We tried to prove that SS30 can inhibit the growth of CD123$^+$ AML cell. Firstly, CD123$^+$ AML cell lines were utilized, as described previously, our data have demonstrated that SS30 could inhibit the growth of CD123$^+$ AML cell (Fig. 3A); Secondly, we tried to use CD34$^+$ cell. The reason is that all the AML cells are differentiated from the CD34$^+$ cells. These CD34$^+$ cells in AML blood are the origin of AML. Thus, to inhibit AML furthest, it is best to inhibit the growth of CD34$^+$ cells. In addition, CD34$^+$ cells were more suitable to test the anti-cancer ability of SS30 than CD123$^+$ AML cell lines as CD34$^+$ cells were isolated from AML patients. Thus, the present study also evaluated the effect of SS30 proliferation and survival inhibition ability of AML blasts from patient specimens. A total of 50 AML patients were divided into a low ($n = 19$), mediate ($n = 13$) and high risk ($n = 18$) group. CD34$^+$ cells were incubated with various concentrations of SS30, thio-random library or PBS, respectively. Subsequently, cell proliferation and survival were assessed using a CCK-8 assay, colony-forming assay and Annexin-V apoptosis detection. As presented in Fig. 4, treatment with SS30

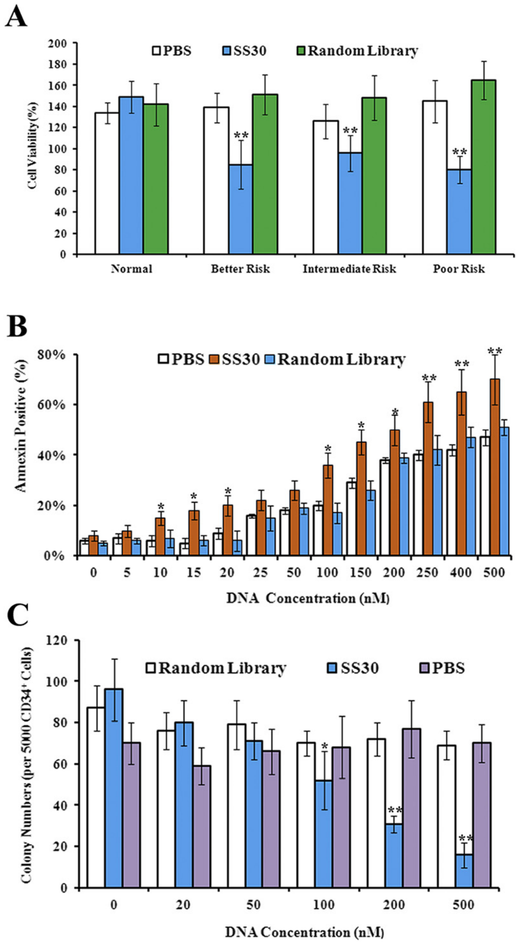

Fig. 4. SS30 inhibits the proliferation and survival of AML blast from patient specimens *in vitro*. (A) Cell Counting Kit-8 assessment of proliferation of AML blasts from patients' samples. AML blasts from patients were treated with SS30, CY30, thio-random library or PBS for 1 h. The cells were then washed with PBS and cultured for a further 36 h. Subsequently, a CCK-8 assay was used to determine cell viability. (B) Analysis of cell apoptosis by Annexin-V. AML blasts from patients were treated with SS30, thio-random library or PBS for 1 h. Cells were washed with PBS and cultured for a further 36 h. The apoptosis of cells were analyzed. (C) Colony forming assay. $CD34^+$ cells from patients were treated with PBS, SS30, or thio-random library. After 14 days, hematopoietic colonies were counted.

resulted in a reduction in the number of AML cells (Fig. 4A), increased apoptosis (Fig. 4B) and a reduced number of colony-forming cells (Fig. 4C). Consistent with the results obtained with the cell lines in Fig. 3, SS30 had a relatively low inhibitory effect on normal $CD34^+$ cells. Furthermore, thio-random ssDNA failed to inhibit AML $CD34^+$ cells and normal $CD34^+$ cells. These observations suggest that SS30 selectively targets AML but spares normal progenitor cells.

3.8. SS30 inhibits $CD123^+$ tumors selectively and has reduced cytotoxicity to normal cells in vivo

It was then evaluated whether SS30 inhibits $CD123^+$ cells *in vivo*. Firstly, it should be verified that whether SS30 could recognize murine CD123. As shown in Supplemental Fig. 3, SS30 could bind to murine CD123 protein as well. Then, the mouse xenograft tumor model was generated by subcutaneously injecting 2×10^7 *in vitro*-propagated Molm-13 cells into BALB/c mice. Tumor-bearing mice were randomly divided into three groups, with six animals in each group: i) Treated with SS30 (2 mg/kg); ii) treated with thio-random library (2 mg/kg); and iii) treated with saline. The weights and tumor volumes of each mouse after agent administration were measured. Whole blood was collected and evaluated for serum markers of organ damage (CK-MB, AST, ALT and BUN) and serum-associated inflammatory cytokines (TNF-α and INF-α). After euthanasia, the heart, liver, kidney, spleen, stomach and lung were collected and subjected to H&E staining. No animal presented with multiple tumors. The maximum diameter exhibited by a single subcutaneous tumor was 15.02 mm. As presented in Fig. 5, SS30 administration resulted in a longer survival time compared with that in the thio-ssDNA and saline groups (Fig. 5A), and the tumor volume in the SS30 group was significantly decreased when compared with that in the thio-ssDNA and saline groups (Fig. 5B)., The tumor volume of SS30 group was 59.21% and 55.40% smaller than that in thio-ssDNA and saline groups, respectively. These results demonstrated the potent anti-cancer effects of SS30 *in vivo*. In addition, The ponderal growth of SS30 group was 84.02% and 75.08% more than that in thio-ssDNA and saline groups, respectively ($P < 0.01$), (Fig. 5C). H&E staining indicated no obvious damage in the SS30 group in comparison to the thio-ssDNA and saline groups (Fig. 5D). Similarly, the serum markers of organs and serum-associated inflammatory cytokines assessed in the SS30 group were similar to those in the thio-ssDNA and saline groups (Fig. 5E). It was thus concluded that SS30 exhibited low cytotoxicity to normal cells and tissues.

3.9. SS30 blocks IL-3-mediated cell proliferation

The present results indicated that SS30 inhibits the proliferation and survival of $CD123^+$ cells *in vitro* and *in vivo*. In a previous study by our group, CY30 was selected as a CD123-binding peptide, which binds to the same site as IL-3. In the present study, SS30, a thiophosphorylated derivative of CY30, was assessed. As IL-3 is able to initiate the proliferation and survival of cells, it was speculated that SS30 inhibits the proliferation and survival of $CD123^+$ cells *via* blocking the interaction of IL-3 and CD123. To evaluate whether SS30 block the interaction of IL-3 and CD123, molecular docking was utilized. As shown in Fig. 6A & B, the 21th, 25th and 27th nucleotide could form hydrogen bond with the 93th, 99th, and 104th amino acid, accordingly. It is reported that the sequence including these amino acids were the main region to recognize IL-3. Thus, it is suspected that SS30 may block the interaction of IL-3 and CD123. Furthermore, to verify this issue, competition assay was used. As presented in Fig. 6C, the viability of $CD123^+$ cell lines (Molm-13, KG-1a, THP-1 and HNT34) declined significantly when treated in a dose-dependent manner. However, SS30 failed to inhibit the proliferation of the $CD123^-$ cell line MV4-11. These results indicated that SS30 may be able to interfere with the binding of IL-3 to CD123 by acting as a competitive inhibitor, thus causing a reduction in cell proliferation.

3.10. SS30 inhibit intracellular signaling in response to IL-3

We have proved that SS30 could interfere the binding between IL-3 and CD123. Further, to further investigate whether SS30 could inhibit intracellular signaling in response to IL-3, Molm-13 cells were incubated with SS30, random DNA (200 nM), or PBS for 2 h. Cells were stained with p-STAT5-Alexa Fluor 647 or p-AKT-Alexa Fluor 647 and analyzed by flow Cytometer and MFI values were recorded. As showed in Fig. 6D, SS30 significantly suppressed IL-3–induced activation of p-STAT5 and p-AKT in a dose-dependent manner. These data further demonstrated that SS30 could block the interaction between IL-3 and CD123.

4. Discussion

The present study reports on the first thioaptamer, SS30, which is able to recognize $CD123^+$ and $CD123^+$ cells with high specificity and affinity, with a Kd of 39.1 nm for CD123 peptide and 287.6 nM for $CD123^+$ AML cells. Furthermore, the anti-proliferative effect of SS30

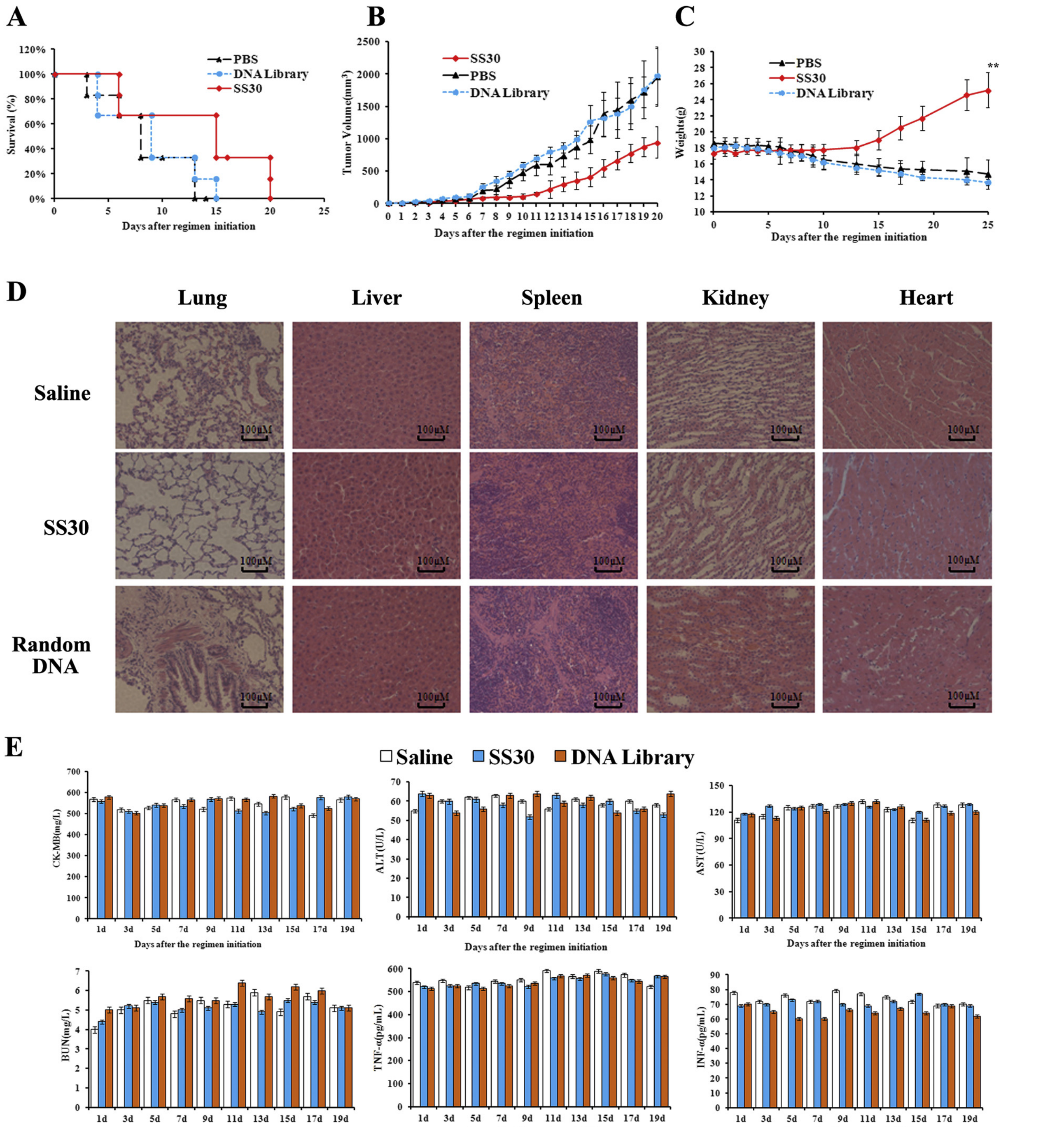

Fig. 5. SS30 inhibits tumor selectively of CD123$^+$ cells and reduces their cytotoxicity to normal cells *in vivo*. A Molm-13 xenograft mouse tumor model was developed by subcutaneous injection of Molm-13 cells in the flank of BALB/c mice. Mice were randomly divided into three groups, which were administered the following regimens by intravenous injection: i) SS30, ii) random DNA library and iii) saline once a day. (A and B) SS30 inhibits CD123$^+$ cell growth *in vivo*. (A) Survival rate of mice after treatment initiation. (B) Tumor volumes of mice after treatment initiation. (C–E). SS30 reduces the cytotoxicity of CD123$^+$ cells to normal tissues *in vivo*. (C) Mice weights up to 25 days after treatment initiation. (D) H&E staining (Microscope magnification: 40 × ; Scale bar: 100 μM). (E) Serology assessment. Serum markers of organ damage were detected. Values are expressed as the mean ± standard deviation of six replicates.

on CD123$^+$ cells lines and AML blasts from patient samples was evaluated, indicating that SS30 was able to inhibit the proliferation of CD123$^+$ cells selectively ($P < 0.01$). SS30 also increased the apoptosis of CD123$^+$ cells when compared with random DNA *in vitro*. Furthermore, SS30 had little toxicity to normal tissues and cells *in vivo*, and significantly prolonged the mean average survival time and decreased the tumor volume of CD123$^+$ tumor-bearing mice *in vivo*.

It is known that IL-3 is the biological ligand for IL-3R, which consists of an α-chain (CD123) and a β-chain [35]. First, IL-3 recognizes CD123 and stimulates the formation of the heterodimer of CD123 and IL-3Rβ [36]. Subsequently, IL-3Rβ initiates downstream signal transduction pathways, including Janus kinase/signal transducer and activator of transcription, mitogen-activated protein kinase and phosphoinositide 3-kinase/Akt, resulting in the proliferation and survival of CD123$^+$ AML cells [37]. The present results indicated that SS30 inhibits the proliferation of CD123$^+$ cells. The target region of SS30 is an extracellular domain of CD123 (TDIECVKDADYSMPAVNNSYCQFG) [38]. This part has been identified as the most immune-dominant peptide epitope

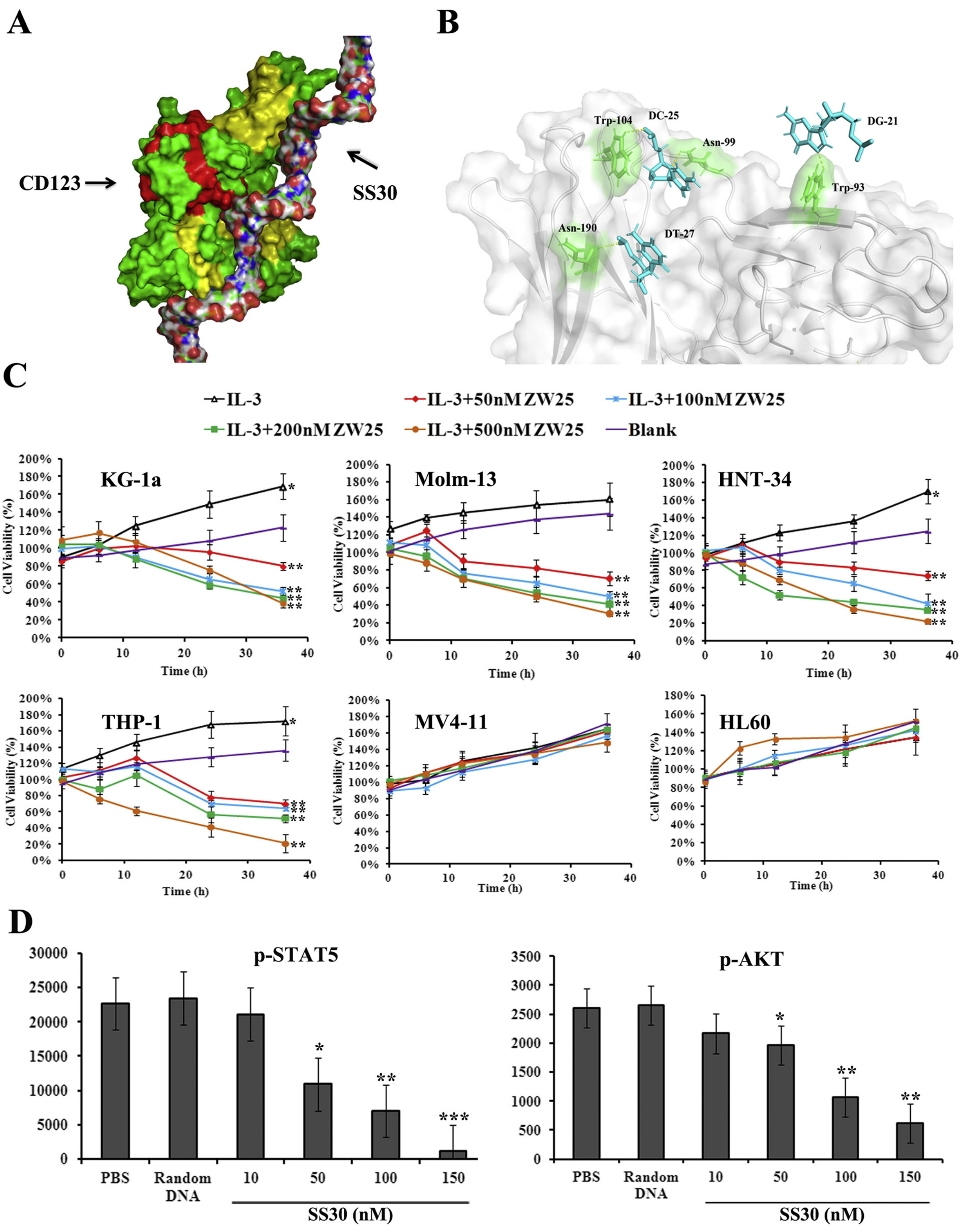

Fig. 6. SS30 competes with IL-3 to bind to CD123 and inhibits the proliferation and signal pathways of CD123$^+$ cell lines. (A) Molecular docking between SS30 and CD123. (B) The hydrogen bond interactions in the docking position. (C) SS30 inhibited IL-3 induced cell proliferation. Molm-13, KG-1a, HNT-34 and THP-1 cells were treated with 50 nM IL-3 and increasing amounts of thioaptamer SS30. Cells were collected at 0, 6, 12, 24 and 36 h, and were analyzed with a Cell Counting Kit-8 kit to evaluate the cell viabilities. (D) Assessment of p-AKT and p-STAT5 expression. Molm-13 cells were incubated with SS30 (10, 50, 100, and 200 nM), random DNA (200 nM), or PBS for 2 h. Cells were cultured for another 12 h and stained with p-STAT5 or p-AKT. Cells were analyzed by flow Cytometer and MFI values were recorded.

within the extracellular domain. The crystal structure of CD123 reveals that the extracellular domain of this protein is always in an extended conformation and it is the key part for IL-3 to recognize CD123. Thus, since SS30 is capable of binding to this peptide, it is predicted that SS30 may compete with IL-3 to bind CD123, which may hamper the IL-3R complex and subsequent downstream signaling, resulting in a reduction in cell proliferation and survival.

As described previously, aptamers are emerging as promising alternative therapeutic ligands that may bind to their molecular targets with high affinity, excellent specificity and low immunogenicity, with a broad prospect in therapeutic applications [39]. However, due to their chemical nature, aptamers are usually susceptible to hydrolysis by nucleases, limiting their potential for pharmaceutical applications [40]. To improve the clinical therapeutic potential of aptamers, various modification methods have been applied [41]. The phosphorothioated modification on the phosphate backbone of nucleic acids is one of the most promising strategies [42]. Phosphorothioate modification may prolong the plasma half-life and allow for dispersion to various tissues within an acceptable time without hampering the binding specificity and affinity of aptamers [43]. However, to the best of our knowledge, no thioaptamer against CD123 has been reported in the literature. In the present study, a novel CD123 thioaptamer based on CY30 was developed; dATP(αS) was used to substitute the regular dATP during PCR amplification for aptamer modification to obtain SS30. In agreement with previous studies on thioaptamers, the present results also indicated that phosphorothioate modification of DNA protected the aptamer from disassembly by nuclease. In addition, the novel CD123 thioaptamer SS30 retained the capability of binding with the CD123 structure in the presence of serum and *in vivo*.

To assess the anticancer effect of SS30 *in vivo*, the mouse xenograft tumor model were randomly divided into three groups, with six in each group: (i) treated with SS30 (2 mg/kg); (ii) treated with thio-random library (2 mg/kg); and (iii) treated with saline. As presented in Fig. 5A, after 11–12 days of the regimen initiation, the survival of PBS and random DNA library group was 0%, whereas SS30 group was 66.67% ($p < 0.01$); After 17 days of the regimen initiation, all mouse died, SS30 resulted in a longer survival time than thio-ssDNA and saline group. However, when compared with anti-cancer ability *in vitro*, the difference *in vivo* in minimal. Meanwhile, the CD123 binding affinity of SS30 was weaker (Kd = 39.1 nM) than CY30. The reasons may be: 1. The phosphorothioate modification may be one factor possibly contributing to the difference in affinity: phosphorothioation may make the aptamer sticky, which presumably interferes with the 3D structure and binding of aptamers; 2. The molecule of SS30 is relative low, after the first *in vivo* circulation, most SS30 may be removed out by kidney filtration; 3. Although SS30 could resist the digestion of nuclease, the internal environment is too complex, SS30 could be consume partly. Thus, the future of our goal is to improve the bio-stability *in vivo* of SS30 by modifying polyethylene glycol (PEG), and to further improve the anti-cancer activity of SS30, we plan to optimize the sequence and phosphorothioate modification of SS30 further, which may improve the binding affinity and pharmacokinetics of SS30. These futures may be useful for the further pre-clinical evaluation for SS30.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.116663.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

All authors listed made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data. In details: Conceived and designed the experiments: Liyu Zhang and Qiao Li; Performed the experiments: Meng Wang, Haibin Wu, Mingyue Duan, Ying Yang, and Fengyu Che; Analyzed the data: Liyu Zhang, Qiao Li, and Haibin Wu; Contributed reagents/materials/analysis tools: Guoxia Wang, Bailing Liu, Wei He, Ying Yang, Qiao Li; Wrote the paper: Liyu Zhang and Meng Wang. All authors were also involved in drafting the manuscript and have given final approval of the version to be published.

Ethics approval and consent to participate

All mouse and rat studies were approved by the Shaanxi Institute of Pediatric Diseases, Xi'an Children's Hospital (Xi'an, China). All animal studies followed the international guidelines on animal welfare and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. In addition, all animal studies complied with the Animal Research: Reporting of *In Vivo* Experiments guidelines and the 2013 American Veterinary Medical Association euthanasia guidelines.

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