

# Ratio-tunable nucleic acid nanohybrids enable precision therapy for esophageal squamous cell carcinoma

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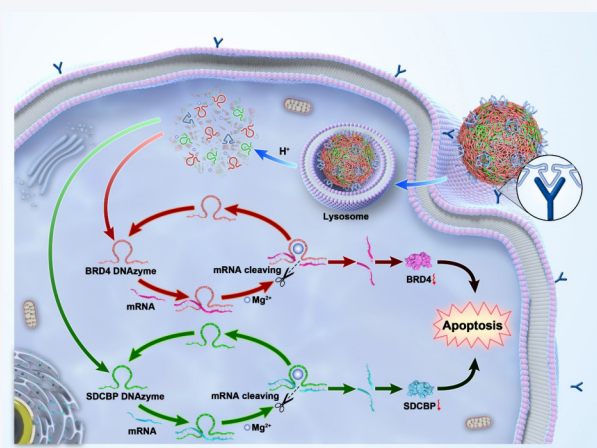
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**ABSTRACT:** Esophageal squamous cell carcinoma (ESCC) progression is strongly associated with the overexpression of bromodomain-containing protein 4 (BRD4) and syndecan-binding protein (SDCBP), identifying them as promising therapeutic targets. Conventional inhibitors, however, are frequently constrained by off-target effects and insufficient tumor specificity. Gene therapy, with nucleic acid drugs as its core, offers the advantage of directly modulating disease-causing gene expression, therefore overcoming these limitations. Therefore, this study aims to develop a multifunctional nucleic acid nanohybrid integrating an ESCC-specific aptamer, BRD4 DNAzyme (BDz), and SDCBP DNAzyme (SDz) for tumor-targeted therapy. This nanohybrid is engineered to respond to the acidic lysosomal environment by releasing BDz and SDz, enabling dual gene silencing, while tumor-specific delivery is mediated by the aptamer. Systematic adjustment of the molar ratios of BDz, SDz, and aptamer A components revealed that a 3:2:1 ratio produced optimal antitumor efficacy. This approach establishes a tunable-ratio dual-DNAzyme delivery platform that maximizes synergistic therapeutic effects while balancing targeted delivery with effective gene silencing, achieving precise dual-target therapy against ESCC. The results provide a base for the development of customizable multifunctional precision gene therapies.



**KEYWORDS:** nucleic acid nanohybrid, gene therapy, dual-gene silencing, DNAzymes, esophageal squamous cell carcinoma

## 1 Introduction

Esophageal cancer ranks as the seventh leading cause of cancer-related mortality worldwide [1]. It comprises two primary pathological subtypes: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC), with ESCC representing nearly 90% of all cases [2, 3]. Current standard treatments for ESCC, such as surgical resection and chemoradiotherapy, are often

associated with chemoresistance and frequent tumor recurrence, along with dose-limiting toxicities [4, 5]. While targeted therapies have shown therapeutic benefits, the identification of novel molecular targets for ESCC remains unexplored [6]. Consequently, it is imperative to identify novel therapeutic targets and formulate effective treatment strategies for ESCC.

Our previous studies have identified bromodomain-containing protein 4 (BRD4) and syndecan binding protein (SDCBP) as key therapeutic targets in ESCC. BRD4, a chromatin reader and critical transcriptional/epigenetic regulator, plays an essential role in oncogene expression [7, 8]. Overexpression of BRD4 has been documented in colorectal [9], breast [10], and lung cancers [11, 12]. The findings demonstrate that BRD4 is substantially upregulated in ESCC tissues relative to normal controls, manifesting at both

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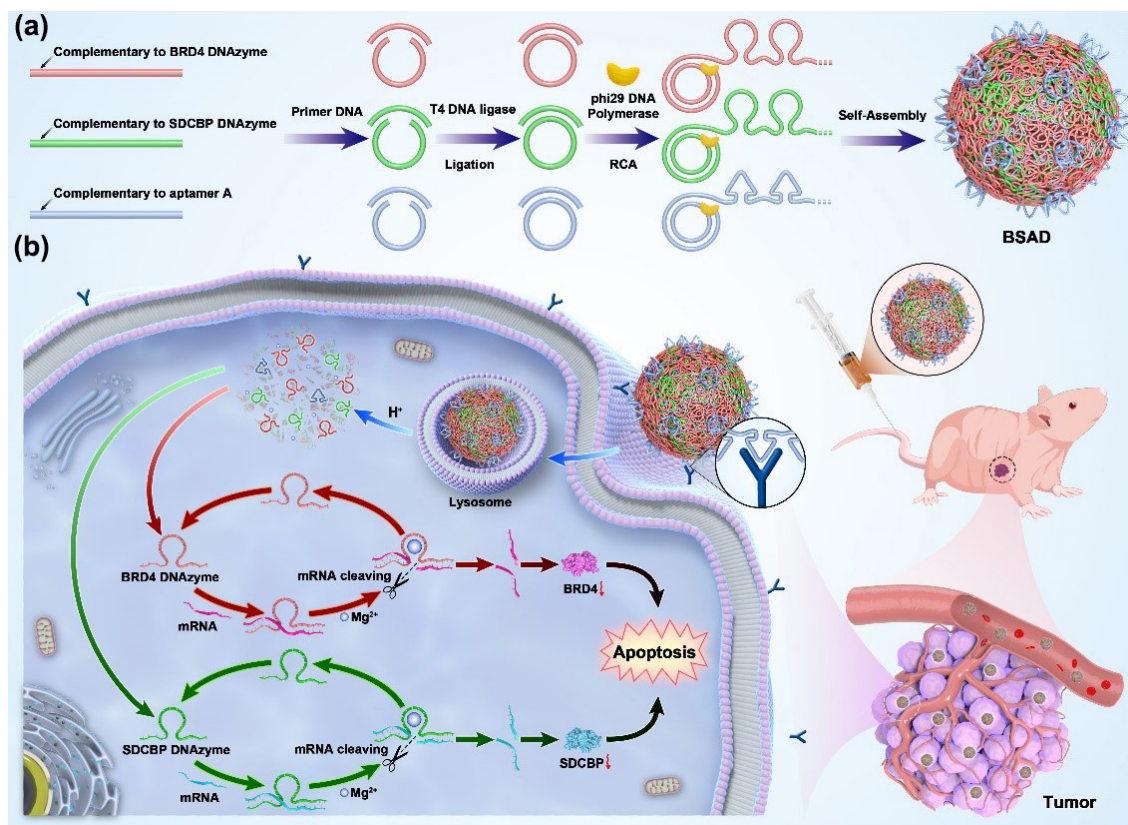
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transcriptional (mRNA) and translational (protein) levels. It interacts with TP73 to increase regulator of chromosome condensation 2 (RCC2) expression, driving tumor proliferation [13]. SDCBP, an adapter protein with two tandem PDZ domains, promotes cancer cell proliferation, migration, invasion, and angiogenesis, facilitating tumorigenesis [14]. It displays oncogenic activity in melanoma [15, 16], breast cancer [17, 18], glioma [19, 20], gastric cancer [21, 22], and other malignancies [23]. Elevated SDCBP expression promotes ESCC progression by binding to epidermal growth factor receptor (EGFR), preserving its membrane localization, and activating the EGFR/PI3K/Akt pathway [24, 25]. These results highlight BRD4 and SDCBP as promising anti-ESCC targets.

Conventional inhibitors frequently encounter limitations such as off-target effects and inadequate tumor specificity. Gene therapy, especially through nucleic acid drugs, addresses these issues by directly targeting disease-causing genes at their origin with high sequence specificity, reducing off-target effects. DNAzymes, a class of programmable catalytic nucleic acid drugs, exemplify these benefits [26]. They hold considerable promise in gene therapy [27, 28], biosensing [29, 30], and theranostic applications [31]. The function of DNAzymes depends on metal ions such as  $Mg^{2+}$ , which are essential for maintaining the correct spatial conformation of their catalytic core and activating their catalytic activity. The recognition arms at both ends of the DNAzyme can specifically bind to the target RNA. Subsequently, mediated by metal ions, the catalytic core is activated and attacks the phosphodiester bond at a specific site on the RNA strand, inducing a hydrolysis reaction at the 2'-OH group of the RNA, ultimately leading to cleavage and degradation of the target RNA at the designated site [32]. Their

high precision, stability, catalytic efficiency, and low cost render them well-suited for oncogene intervention [33]. However, clinical translation of DNAzyme-based therapies is hindered by challenges such as limited intracellular delivery efficiency and the inherent constraint of targeting a single gene, an approach that may be inadequate given cancer's regulation through complex, multi-gene networks and the heterogeneous expression of target genes [34]. Therefore, advancing safe and efficient multi-gene silencing DNAzyme platforms capable of synergistic action and accommodating target expression variability is crucial for achieving effective cancer therapy [35, 36].

Taking into account these considerations, a proportionally tunable self-assembled nucleic acid nanohybrid was developed, enabling both active targeting of ESCC cells and a dual-gene silencing mechanism for inducing antitumor effects. BRD4 DNAzyme (BDz), SDCBP DNAzyme (SDz), and an ESCC-specific targeting aptamer were co-assembled into the nanohybrids at predetermined ratios while employing rolling circle amplification (RCA) (Scheme 1(a)). Following aptamer-mediated tumor-targeted delivery, the nanohybrids undergo dissociation within the acidic lysosomal microenvironment, leading to the disassembly of their magnesium pyrophosphate framework and subsequent release of BDz, SDz, and  $Mg$  ions. The latter serves as a cofactor to activate the DNAzymes' targeted mRNA cleavage, enabling dual-gene silencing, thus inducing tumor cell apoptosis (Scheme 1(b)). Notably, this study addresses tumor heterogeneity challenges by optimizing the molar ratios of BDz/SDz/Aptamer to maximize synergistic therapeutic efficacy, while employing the self-assembled architecture to improve structural stability, resist nuclease degradation, and maintain therapeutic activity.



**Scheme 1** Illustration of (a) the synthesis of the ratio-tunable, multifunctional nucleic acid nanohybrid (BSAD) and (b) BSAD-enabled efficient dual-gene silencing and drug delivery in cancer cells.

## 2 Results and discussion

### 2.1 Preparation and characterization of nucleic acid nanohybrids

The cleavage activities of BDz and SDz were initially assessed via polyacrylamide gel electrophoresis (PAGE) using their respective target substrates, B-Sub for BDz and S-Sub for SDz. As presented in Figs. S1 and S2 in the Electronic Supplementary Material (ESM),

both DNAzymes showed significant cleavage efficiency under 5 mM  $Mg^{2+}$  conditions, with BDz achieving  $67.82\% \pm 7.28\%$  cleavage of B-Sub, and SDz achieving  $88.43\% \pm 5.41\%$  cleavage of S-Sub. To further investigate the cleavage kinetics, fluorescence resonance energy transfer (FRET) assays were conducted under 5 mM  $Mg^{2+}$  conditions. As illustrated in Fig. 1(a), fluorescence quenching occurs when the two fluorophores are in close proximity, whereas an increase in the inter-dye distance leads to fluorescence recovery [37, 38]. As shown in Fig. 1(b),  $Mg^{2+}$  induced

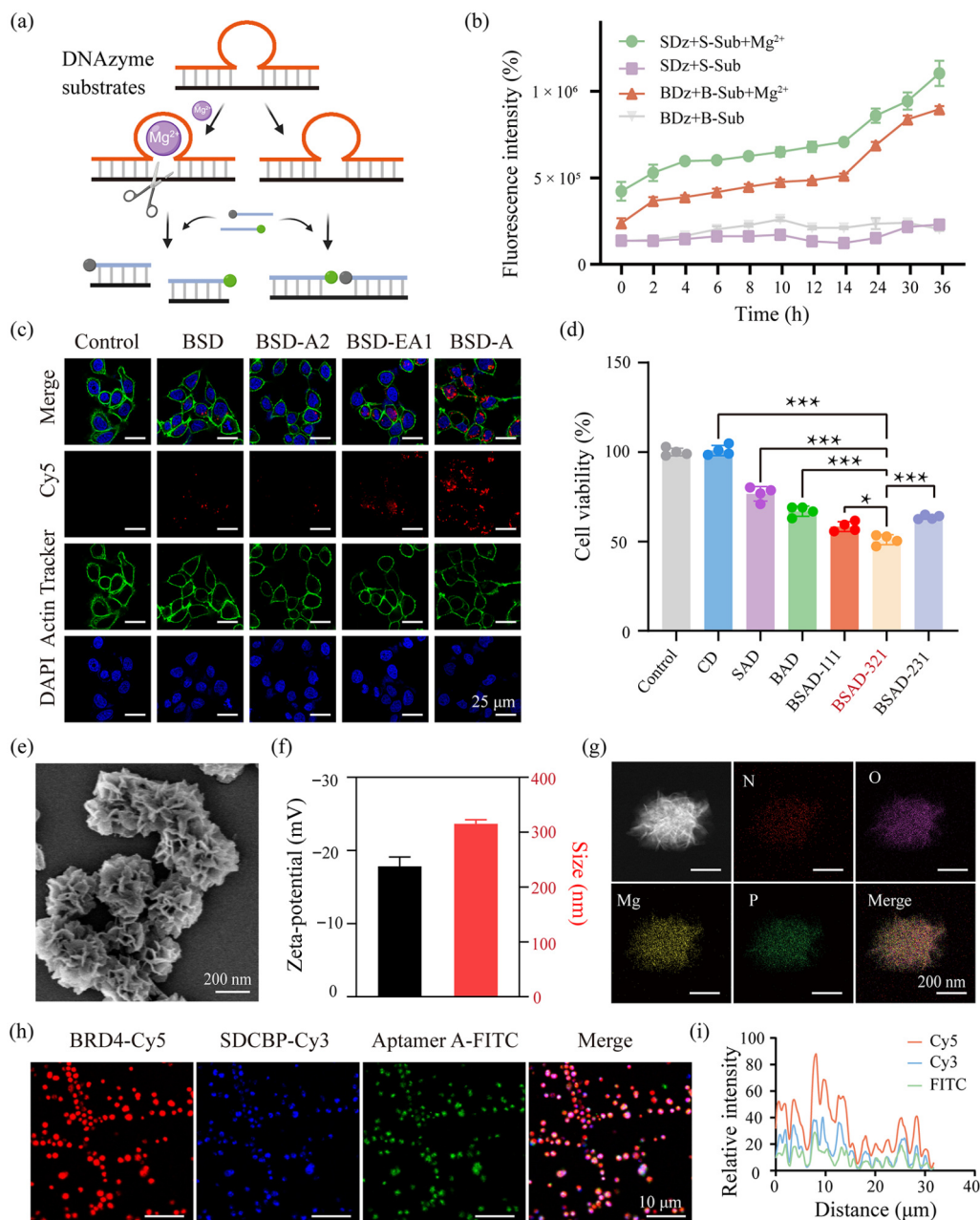


**Figure 1** Preparation and characterization of nucleic acid nanohybrids. (a) Schematic illustrating the catalytic mechanism of BSAD-integrated DNAzymes:  $Mg^{2+}$ -dependent cleavage of target mRNA substrates monitored via FRET. (b) Fluorescence kinetics curves ( $n = 3$ ) demonstrating catalytic cleavage activity of BRD4 DNAzyme (BDz) and SDCBP DNAzyme (SDz) on their respective RNA substrates over a 36 h period. (c) CLSM images of KYSE450 cells incubated with different nanohybrids for 4 h ( $n = 3$ ). Scale bars: 25  $\mu m$ . (d) Viability of KYSE450 cells after incubating with different samples for 48 h ( $n = 4$ ). (e) SEM image of BSAD. Scale bar: 200 nm. (f) Particle size and zeta potential of BSAD ( $n = 3$ ). (g) EDS elemental mapping of BSAD. Scale bar: 200 nm. (h) CLSM images of BSAD after synthesis with the BDz labeled with Cy5, the SDz labeled with Cy3, and the aptamer A labeled with FITC (scale bars: 10  $\mu m$ ). (i) Colocalization analysis of the CLSM images in (h). Data are presented as mean  $\pm$  standard deviation (SD). Group comparisons were performed using one-way analysis of variance (ANOVA), with statistical significance defined as: \* $P < 0.05$ ; \*\*\* $P < 0.001$ .

a continuous fluorescence increase in fluorescence intensity over time, reflecting time-dependent substrate cleavage by the DNAzyme. In comparison,  $Mg^{2+}$  omission resulted in persistent fluorescence quenching, demonstrating catalytic incompetence. These findings confirm that BDz and SDz show strong intrinsic RNA cleavage activity.

To increase tumor-targeting specificity and minimize potential off-target effects, three aptamers, designated A, A2, and EA1, were selected as candidate targeting ligands. These aptamers have been previously reported [39–41] to possess binding affinity for esophageal cancer cells. The nucleic acid nanohybrids were synthesized by first generating circular DNA templates for each component, followed by mixing and subjecting them to RCA (all sequences are listed in Tables S1–S3 in the ESM). Following the synthesis of circular DNA templates for the selected aptamers, co-RCA was performed with circular DNA templates for BDz and SDz to generate the corresponding BSD-A, BSD-A2, and BSD-EA1 nanohybrids. A control nanohybrid containing only BDz and SDz, without any aptamer, was synthesized and designated BSD. Transmission electron microscopy (TEM) was employed to characterize the nanohybrid structures, confirming successful fabrication (Fig. S3 in the ESM). Cellular uptake and localization were examined using confocal laser scanning microscopy (CLSM). BSD-A displayed the highest targeting capability toward Kyse450 cells, with an intracellular Cy5 signal intensity approximately 3.5-fold greater than that of the aptamer-free BSD ( $P < 0.001$ ; Fig. 1(c) and Fig. S4 in the ESM). These findings indicate that the aptamer A-integrated nanohybrid (BSD-A) displays improved targeting efficiency toward Kyse450 cells and was therefore selected as the targeting ligand for further experiments to increase precision delivery in esophageal cancer therapy.

In pharmaceutical development, variability in the expression levels of different therapeutic targets can compromise therapeutic efficacy. Fixed stoichiometric ratios may result in inadequate silencing of specific targets or unintended off-target effects. Ratio-tunable platforms provide the flexibility to optimize the silencing efficiency of each target individually. Interpatient heterogeneity in tumor target expression necessitates the development of personalized therapeutic strategies. Ratio tunability enables such customization by adjusting strand proportions to match individual molecular profiles, increasing therapeutic precision. In the following experiments, the ratios of the aptamer strand to the two DNAzyme silencing strands were precisely controlled to maximize synergistic effects, optimize the balance between targeting and silencing, and achieve precise multi-target therapy. Ternary nanohybrids were synthesized at three distinct molar ratios of BDz, SDz, and aptamer A: 1:1:1, 2:3:1, and 3:2:1, designated BSAD-111, BSAD-231, and BSAD-321, respectively. For comparison, single-target nanohybrids containing only one therapeutic strand (BDz or SDz) conjugated to aptamer A were also synthesized as controls and designated BAD (BDz + aptamer A) and SAD (SDz + aptamer A). Control DNAzymes containing mutations in their catalytic core sequences were designed, and the corresponding control nanohybrids were designated CD. The synthesized nanohybrids were first characterized. As shown in Figs. S5(a)–S5(d) in the ESM, electrophoretic analysis of the circularization products on a 2% agarose gel revealed that the circularized DNA displayed lower electrophoretic mobility compared to the linear form, confirming successful circular DNA formation. The RCA products, owing to their high molecular weight, remained near the gel wells (Fig. S6 in

the ESM), with all nanohybrid types displaying similar migration patterns at the gel top. Following structural characterization via TEM (Fig. S7 in the ESM), cytotoxicity assessment of the proportionally formulated nanohybrids was performed through cell counting kit-8 (CCK-8) assays (Fig. 1(d)). As anticipated, control Kyse450 cells showed the highest viability. Dual-target nanohybrids demonstrated significantly greater cytotoxicity compared to their single-target counterparts. Among all tested formulations, BSAD-321 produced the most significant cytotoxic effect on Kyse450 tumor cells, yielding the lowest observed cell survival rate. To further validate these results and directly assess cell viability, Kyse450 cells treated with various nanohybrid formulations were co-stained with Calcein-AM (green fluorescence, live cells) and propidium iodide (PI, red fluorescence, dead cells), followed by fluorescence microscopy (Fig. S8 in the ESM). In agreement with the CCK-8 assay results, BSAD-321-treated cells showed the most intense red fluorescence, confirming its strong cytotoxic activity. In comparison, cells exposed to other formulations predominantly displayed green fluorescence or weaker red fluorescence, indicating higher survival rates. Based on its increased cytotoxic efficacy against Kyse450 cells in both the CCK-8 and live-dead assays, the BSAD-321 formulation was selected for all the next experiments. This optimized nanohybrid, hereafter designated as BSAD, displayed the highest tumoricidal activity among all evaluated configurations.

The morphology of BSAD was examined using TEM (Fig. 1(e)), revealing a spherical, flower-like structure. Dynamic light scattering (DLS) analysis revealed that BSAD possessed an average hydrodynamic diameter of approximately 320 nm (Fig. 1(f) and Fig. S9 in the ESM), aligning with the size measurements obtained from SEM and TEM observations. The zeta potential of BSAD was approximately  $-18$  mV. Elemental mapping and energy-dispersive X-ray spectroscopy (EDS) (Fig. 1(g) and Fig. S10 in the ESM) confirmed the presence of nitrogen (N), oxygen (O), magnesium (Mg), and phosphorus (P), consistent with the composition of nanohybrids containing a magnesium pyrophosphate core. The synthesized BSAD was functionalized with Cy5-labeled BDz, Cy3-labeled SDz, and FITC-labeled aptamer A. Confocal microscopy analysis confirmed colocalization of the three components (Figs. 1(h) and 1(i)), indicating their spatial association within the nanohybrids. Magnesium pyrophosphate ( $Mg_2P_2O_7$ ) constitutes the core framework of the BSAD nanohybrid, which disintegrates in the acidic lysosomal environment, simultaneously releasing  $Mg^{2+}$  and DNAzymes. Scanning electron microscopy (SEM) imaging (Fig. S11 in the ESM) revealed a pronounced collapse of the BSAD morphology under acidic conditions. To quantitatively assess its  $Mg^{2+}$  release capability at different pH values, inductively coupled plasma mass spectrometry (ICP-MS) was further employed. As shown in Fig. S12 in the ESM, the concentration of  $Mg^{2+}$  released from BSAD at pH 5.5 was significantly higher than that in the pH 7.4 group, confirming that acidic conditions trigger the disintegration of its inorganic core, consistent with the morphological observations from SEM. Following confirmation of the structural disassembly, the functional activity under acid-responsive conditions was evaluated via PAGE (Fig. S13 in the ESM). BSAD exhibited efficient RNA substrate cleavage at pH 5.5, indicating that the acid-triggered disassembly is accompanied by the functional release of DNAzymes.

To evaluate the stability of BSAD as a delivery platform, a series of *in vitro* tests were conducted. Agarose gel electrophoresis results

(Fig. S14 in the ESM) demonstrated that BSAD maintained structural integrity under 10% FBS, 2 U/mL DNase I, and 95 °C heat treatment. Long-term stability monitoring (Fig. S15 in the ESM) showed negligible changes in particle size over 8 days. In addition, *in vitro* hemolysis assays confirmed the favorable hemocompatibility of BSAD (Fig. S16 in the ESM), supporting its potential for *in vivo* application.

## 2.2 Cellular uptake and gene silencing efficacy of BSAD

Targeted drug delivery is central to achieving precision therapy. BSAD incorporates aptamers that selectively recognize esophageal cancer cells, imparting tumor-targeting capability. This specificity reduces off-target interactions with non-malignant tissues, an essential consideration for therapeutic safety. Previously, cellular

uptake of BSAD by KYSE450 cells was confirmed via confocal microscopy. In the present study, uptake was further quantified using flow cytometry (Fig. 2(a), and Fig. S17 in the ESM). The results demonstrated that the aptamer-functionalized BSAD achieved efficient internalization by KYSE450 cells within 4 h, whereas aptamer-deficient nanohybrids (BSD) showed significantly reduced uptake, consistent with the confocal microscopy findings. To elucidate the internalization pathway, lysosomes were labeled with Lyso-Tracker. After 1 h of treatment, Cy5-BSAD (red) signals extensively co-localized with Lyso-Tracker (green), indicating predominant lysosomal localization (Fig. S18 in the ESM). At 3 h, the co-localization significantly decreased, suggesting that BSAD underwent lysosomal escape.

Quantitative real-time polymerase chain reaction (qRT-PCR)

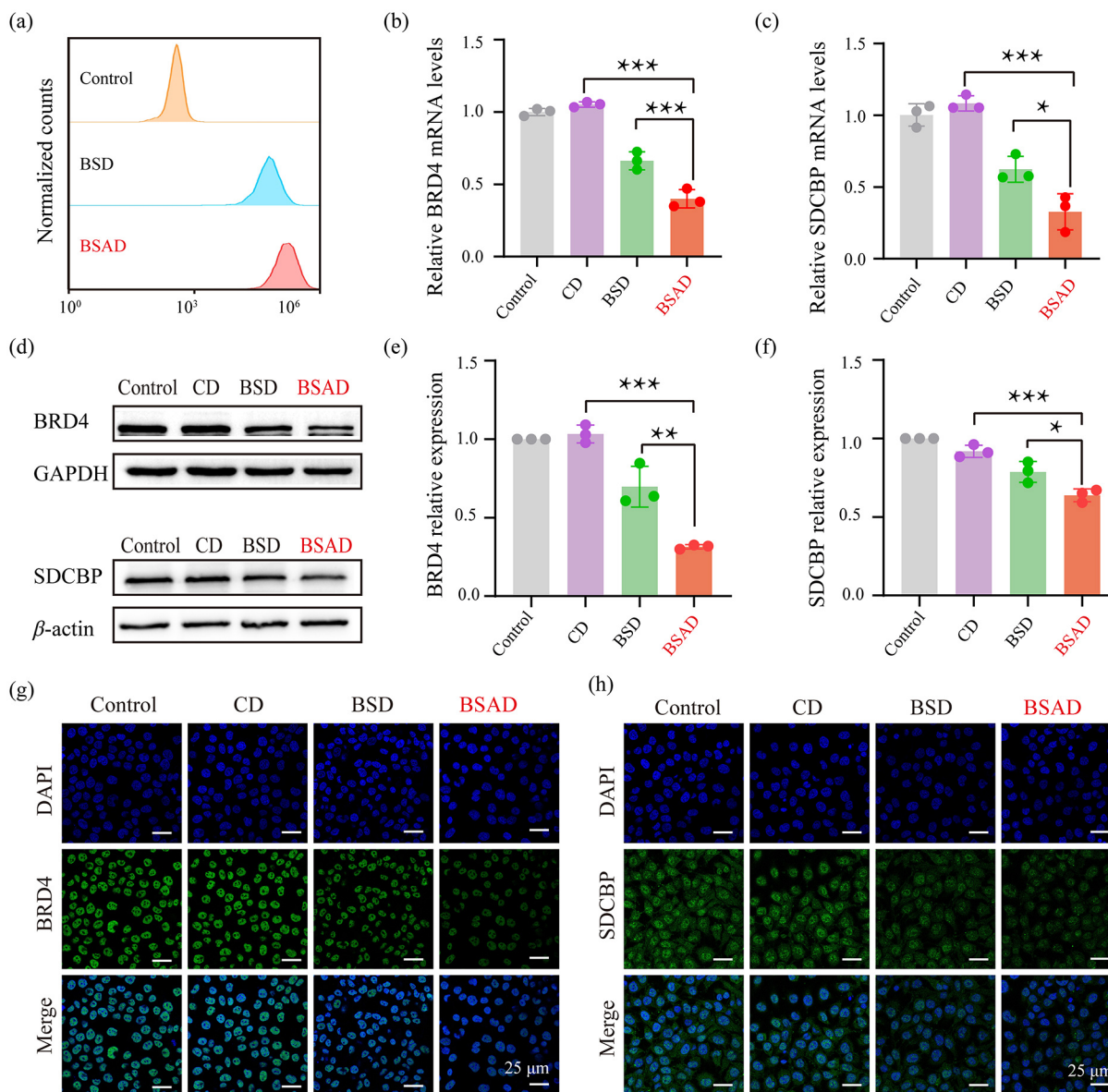


**Figure 2** Cellular uptake and gene silencing efficacy of BSAD. (a) Flow cytometry analysis of KYSE450 cells treated with Cy5-BSD and Cy5-BSAD for 4 h. qRT-PCR analysis of (b) BRD4 and (c) SDCBP mRNA in KYSE450 cells after 24 h of different treatments. (d) Western blot analysis of BRD4 and SDCBP proteins in KYSE450 cells after 48 h of different treatments. Semi-quantitative analysis of (e) BRD4 and (f) SDCBP protein levels in treated KYSE450 cells by Western blot: (g) Representative images of BRD4 immunofluorescence (green) in KYSE450 cells. Nuclei are stained with 4',6-diamidino-2-phenylindole (DAPI, blue). Scale bar: 25  $\mu$ m. (h) Representative images of SDCBP immunofluorescence (green) in KYSE450 cells. Nuclei are stained with DAPI (blue). Scale bars: 25  $\mu$ m. Data are presented as mean  $\pm$  SD ( $n = 3$ ). Differences between groups were assessed using one-way analysis of variance (ANOVA). Statistical significance was defined as follows: \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

was performed to evaluate BRD4 and SDCBP mRNA expressions in KYSE450 cells following various treatments (Figs. 2(b) and 2(c)) (primers used are listed in Table S4 in the ESM). BSAD treatment resulted in a  $60.02\% \pm 5.62\%$  reduction in BRD4 mRNA and a  $67.46\% \pm 6.66\%$  reduction in SDCBP mRNA levels. In comparison, BSD-treated cells displayed less significant decrease, indicating that the DNAzyme component effectively mediated mRNA knockdown, with the aptamer contributing significantly to this effect. As a control, cells exposed to CD (DNAzyme with a mutated catalytic core sequence) displayed negligible changes in BRD4 and SDCBP expression, underscoring the essential role of the intact catalytic core for enzymatic activity. Western blot analysis and semi-quantitative evaluation further corroborated the inhibitory effect of BSAD on BRD4 and SDCBP protein expression. As shown in Figs. 2(d)–2(f), no significant difference was observed between the CD group and the control group, indicating that the nanohybrid scaffold alone does not influence protein expression. In the BSD group, a significant reduction in BRD4 and SDCBP protein levels was detected, attributable to DNAzyme-mediated mRNA cleavage via its intact catalytic core. Treatment of KYSE450 cells with BSAD resulted in a more pronounced suppression of BRD4 and SDCBP protein expression, which is ascribed to aptamer-mediated tumor cell targeting. Immunofluorescence analysis (Figs. 2(g), 2(h), and Fig. S19 in the ESM) further confirmed these findings, as BSAD-treated cells showed significantly diminished green fluorescence signals compared with the control group, corresponding to BRD4 and SDCBP. These results indicate that BSAD-mediated gene silencing is driven by the catalytic activity of BDz and SDz, which cleave their respective target mRNAs, and that aptamer-guided delivery increases silencing efficiency by ensuring selective uptake into tumor cells.

### 2.3 *In vitro* therapeutic efficacy of BSAD

Having established the efficient cellular uptake and potent gene silencing capability of BSAD, we next evaluated its anti-tumor efficacy *in vitro*. The inhibitory effect of BSAD on cell proliferation was evaluated using the CCK-8 assay (Fig. 3(a)). KYSE450 cells were incubated for 72 h with BSAD at concentrations ranging from 0 to 200 nM, and cell viability was assessed by measuring absorbance at 450 nm. Analysis of the results demonstrated that BSAD effectively suppressed cell proliferation in a concentration-dependent manner. The half-maximal inhibitory concentration ( $IC_{50}$ ) for KYSE450 cells was determined to be 112 nM.

The impact of BSAD on the proliferative capacity of KYSE450 cells was evaluated using the EdU incorporation assay and the plate colony formation assay. The EdU assay measures the proportion of cells undergoing DNA synthesis. As shown in Fig. 3(b) and Fig. S21(a) in the ESM, treatment with either BAD or SAD significantly reduced cell proliferation compared with the control group, whereas BSAD treatment produced the most significant suppression, resulting in the lowest proportion of EdU-positive cells. Consistent with these findings, the colony formation assay (schematic in Fig. 3(c), results in Fig. 3(d) and Fig. S21(b) in the ESM) demonstrated that BSAD significantly impaired the long-term proliferative potential of KYSE450 cells, yielding fewer and smaller colonies and a significantly reduced colony formation rate relative to BAD- or SAD-treated groups. Both assays confirmed that BSAD exerted the most potent inhibitory effect on KYSE450 cell proliferation, surpassing the efficacy of the single-target nanohybrids.

Apoptosis induction by BSAD in KYSE450 cells was assessed using Annexin V/PI dual staining followed by flow cytometric analysis. Annexin V selectively binds to phosphatidylserine exposed on the outer leaflet of the plasma membrane during early apoptosis, marking cells with intact membranes. PI, which penetrates cells with compromised membranes and binds to nucleic acids, identifies late apoptotic and necrotic cells. This combination allows discrimination among viable, early apoptotic, and late apoptotic/necrotic cells. As shown in Fig. 3(e) and Fig. S21(c) in the ESM, BSAD treatment resulted in the highest apoptosis rate, whereas BAD and SAD treatments produced comparatively lower rates under identical conditions. These findings indicate that the dual-target BSAD nanohybrid significantly elevates apoptosis induction in tumor cells through simultaneous suppression of BRD4 and SDCBP expression.

Cell migration and invasion capabilities were systematically evaluated through wound healing assays and Transwell experiments. The progression of wound healing was monitored at three time points (0, 12, and 24 h) using microscopic imaging. As shown in Figs. 3(f) and 3(g), after 24 h, the wound healing rate was reduced by 34.45% and 39.90% in the SAD and BAD groups, respectively, compared with the CD group, with the most pronounced reduction (57.03%) observed in the BSAD group. These findings indicate that BSAD significantly suppresses KYSE450 cell migration, surpassing the efficacy of single-target therapies (BAD or SAD). The Transwell migration assay further confirmed this result: BSAD exhibited a significantly stronger inhibitory effect on cell migration than single-target nanohybrid group (Fig. S20 in the ESM). The invasion-inhibitory potential of BSAD was confirmed through Transwell invasion assays, where the number of Matrigel-penetrating cells was significantly lower than in the control group (Fig. 3(h) and Fig. S21(d) in the ESM), with inhibition being significantly greater than that achieved by BAD or SAD. The experimental findings indicate that, at the optimized ratio, this tunable nanohybrid displays potent antitumor activity, efficiently suppressing Kyse450 cell proliferation and migration, and surpassing the efficacy of single-target nanohybrids.

### 2.4 *In vivo* therapeutic efficacy of BSAD

Building on the promising *in vitro* therapeutic performance of BSAD, the *in vivo* tumor-targeting capability and antitumor efficacy were then examined (Fig. 4(a)). The tumor-targeting ability was first assessed in mice bearing Kyse450 xenografts, which were intravenously administered Cy5-labeled BSAD, Cy5-labeled BSD, or free Cy5 via the tail vein. Whole-body fluorescence imaging was performed at predetermined time points post-injection (Fig. 4(b) and Fig. S22(a) in the ESM). At 4 h post-injection, the Cy5-BSAD group displayed strong fluorescence signals concentrated in tumor regions, whereas the other two groups represented minimal tumor localization. At the experimental endpoint (24 h post-treatment), major organs and tumor tissues were harvested for *ex vivo* imaging to assess drug signal intensity. Although inevitable hepatic and renal accumulation was observed, tumors in the Cy5-BSAD group exhibited ~4-fold greater fluorescence signal intensity compared to the Cy5-BSD group (Fig. 4(c) and Fig. S22(b) in the ESM). These findings confirm that aptamer functionalization of nanohybrids significantly improves their *in vivo* tumor-targeting specificity.

Next, the *in vivo* antitumor efficacy of BSAD was evaluated. Two weeks post-implantation, mice received tail vein injections of the respective treatments every two days, with body weight and tumor

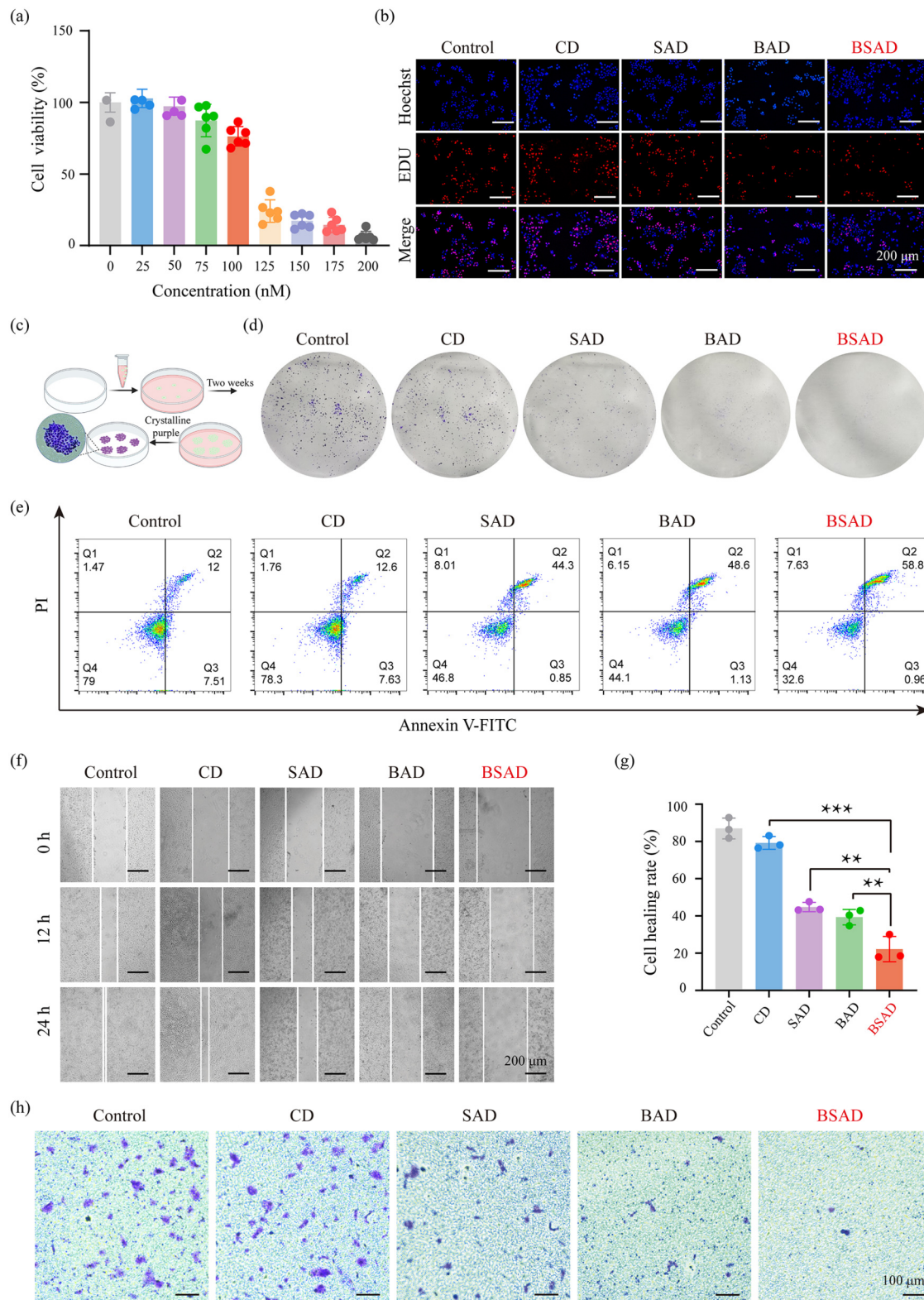


**Figure 3** *In vitro* therapeutic efficacy of BSAD. (a) Cell viability curve of KYSE450 cells treated for 72 h with increasing concentrations of BSAD ( $n = 6$ ). (b) EdU proliferation assay of KYSE450 cells after 24 h exposure to various treatments ( $n = 3$ ). Scale bars: 200  $\mu\text{m}$ . (c) Schematic diagram of colony formation assay. (d) Colony formation assay of KYSE450 cells after 14 days under distinct treatment conditions ( $n = 3$ ). (e) Apoptosis analysis by Annexin V/PI staining after 48 h of different treatments ( $n = 3$ ). (f) Representative images of wound healing assay in differently treated KYSE450 cells ( $n = 3$ ). Scale bars: 200  $\mu\text{m}$ . (g) Semi-quantitative analysis of cell migration capacity (cell healing rate). (h) Transwell invasion assay of KYSE450 cells after 48 h of different treatments ( $n = 3$ ). Scale bars: 100  $\mu\text{m}$ . Data are presented as mean  $\pm$  SD. Group comparisons were performed using one-way analysis of variance (ANOVA), with statistical significance defined as: \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . EdU (5-ethynyl-2'-deoxyuridine) is a recently developed thymidine analogue which serves as a replacement for thymidine during DNA synthesis, becoming incorporated into newly formed DNA strands.

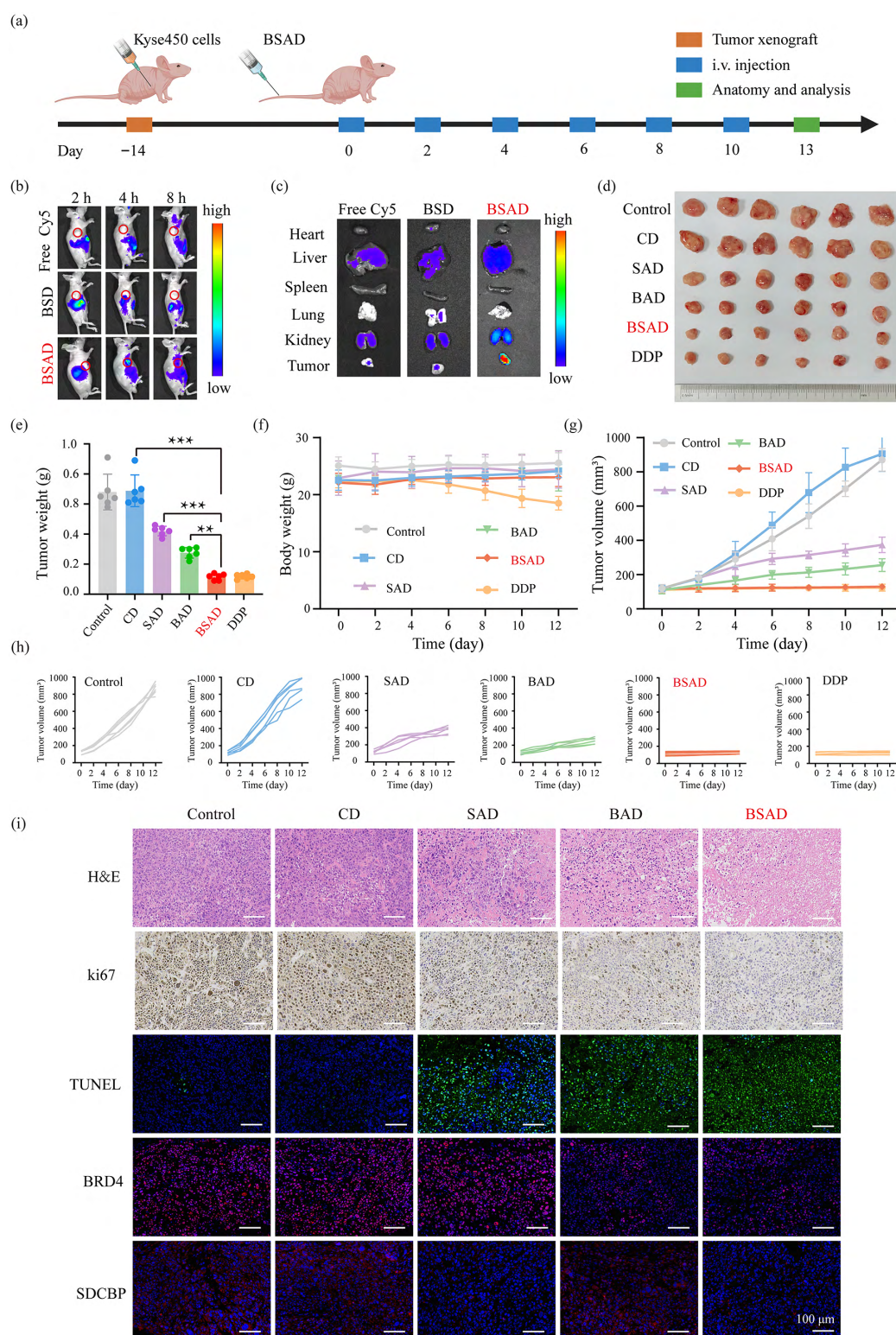


**Figure 4** *In vivo* therapeutic efficacy of BSAD. (a) Schematic diagram of the therapeutic regimen in tumor-bearing mice. (b) *In vivo* fluorescence imaging for evaluating tumor-targeting capability of BSAD dynamic distribution of Cy5-labeled BSAD probes in tumor-bearing mice at critical time points (2, 4, 8 h) post-administration. (c) *Ex vivo* fluorescence imaging of dissected tumors and major organs 24 h post-injection of Cy5-labeled BSAD. (d) Representative images of xenograft tumors from nude mice across treatment groups at the experimental endpoint, and (e) tumor weight (n = 6). (f) Body mass measurements for the various mouse cohorts throughout the treatment period (n = 6). (g) Tumor growth progression monitored in each experimental group during treatment (n = 6). (h) Individual tumor growth curves in mice after different treatments. (i) Representative images of tumor sections from different treatment groups: row 1: H&E staining; row 2: Ki67 immunohistochemical staining; row 3: TUNEL fluorescence staining; row 4: immunofluorescence staining for BRD4; row 5: immunofluorescence staining for SDCBP (scale bar: 100  $\mu$ m, applies to all panels). Data are presented as mean  $\pm$  SD. Group comparisons were performed using one-way analysis of variance (ANOVA), with statistical significance defined as: \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . DDP, cisplatin.

volume monitored throughout the study. At the end of treatment, tumors were excised for imaging and weighing (Figs. 4(d) and 4(e)).

During the treatment period, mice in the cisplatin positive control group displayed significant weight loss and emaciation, indicative of drug-induced toxicity (Fig. 4(f)). In comparison, no significant weight loss was observed in the control group or mice treated with either dual-target or single-target nanohybrids. Tumor growth curves revealed significant growth suppression in all nanohybrid-treated groups, with BSAD producing greater tumor inhibition than BAD or SAD (Figs. 4(g) and 4(h)). The antitumor efficacy of BSAD was comparable to that of cisplatin, but with a more favorable safety profile.

Histological analysis was conducted to further assess the *in vivo* therapeutic effects of BSAD (Fig. 4(i)). Hematoxylin and eosin (H&E) staining revealed that (Fig. 4(i), row 1), compared with the control group, tumor sections from the BSAD-treated group showed extensive fibrosis and significant architectural disruption, characterized by loss of cellular morphology and widespread nuclear disintegration. In comparison, tumors from the BAD- and SAD-treated groups showed partial fibrosis with some intact tumor cells remaining. These results indicate that BSAD induces tumor cell death through synergistic mechanisms, resulting in tissue damage significantly greater than that caused by single-target nanohybrids, confirming its improved therapeutic efficacy from a pathological standpoint.

To evaluate tumor cell proliferation, Ki67 immunohistochemistry (IHC) was performed. The control group displayed high proliferative activity, with densely packed brown-stained nuclei (Fig. 4(i), row 2). BSAD-treated tumors showed a pronounced reduction in proliferation, with only sporadic Ki67-positive nuclei. BAD and SAD treatment also reduced proliferation but to a lesser extent than BSAD, consistent with the anti-proliferative effects observed in *in vitro* EdU and colony formation assays.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) immunofluorescence analysis (Fig. 4(i), row 3, and Fig. S23(a) in the ESM) revealed a significant increase in green fluorescence-positive cells in the BSAD group, indicating elevated apoptosis. Moderate apoptotic activity was observed in the BAD and SAD groups, in agreement with the H&E staining results.

To elucidate the mechanism underlying tumor growth inhibition, BRD4 and SDCBP protein levels in tumor tissues were analyzed via immunofluorescence. Compared to the control group, BRD4 expression was reduced in the BAD and BSAD groups (Fig. 4(i), row 4, and Fig. S23(b) in the ESM), while SDCBP expression was decreased in the SAD and BSAD groups (Fig. 4(i), row 5, and Fig. S23(c) in the ESM). The concurrent downregulation of both BRD4 and SDCBP in the BSAD group reflects the targeted silencing activity of the dual-target nanohybrid. These results confirm that BSAD exerts its antitumor effects by suppressing BRD4 and SDCBP expression, promoting increased apoptosis and a significant therapeutic response *in vivo*.

## 2.5 *In vivo* biosafety of BSAD

Systemic biosafety of the therapeutic regimen was assessed by collecting major organs (heart, liver, spleen, lungs, kidneys) and peripheral blood from two groups: (i) Kyse450 tumor-bearing nude mice following antitumor efficacy studies, and (ii) tumor-free nude mice administered equivalent drug doses. Histopathological evaluation using H&E staining showed no significant structural abnormalities in any organs across all groups (Fig. 5(a) and Fig. S24

in the ESM). In parallel, comprehensive hematological analyses, including complete blood count (CBC) and serum biochemistry, revealed no indicators of drug-induced toxicity (Figs. 5(b)–5(k) and Figs. S25 and S26 in the ESM). These results collectively confirm the favorable biosafety profile of the BSAD formulation.

## 3 Conclusions

In conclusion, this study demonstrates that BSAD, a ratio-tunable self-assembled nucleic acid nanohybrid, can be precisely engineered to co-deliver therapeutic DNAzymes targeting BRD4 and SDCBP in ESCC. Integration of an ESCC-specific aptamer with BDz and SDz at an optimized molar ratio (3:2:1) enables tumor-targeted delivery and acid-responsive DNAzyme release. This dual-gene silencing strategy modulates the TP73/RCC2 and EGFR/PI3K/Akt pathways, effectively suppressing ESCC proliferation, migration, and tumor growth *in vitro* and *in vivo*, while maintaining a favorable safety profile. The tunable-ratio design allows adaptation to heterogeneous target expression, enhancing therapeutic synergy, targeting specificity, and gene silencing efficiency. These findings establish BSAD as a versatile platform for multi-gene intervention and present a novel, ratio-adjustable dual-DNAzyme therapeutic strategy for advancing multifunctional precision gene therapies.

Our designed platform features a high degree of modularity and programmability, in which the targeting aptamers and therapeutic nucleic acid sequences can be readily replaced to address other diseases. Additionally, the two independent therapeutic components can be directed toward genes with synergistic effects, thereby further enhancing the therapeutic efficacy. Moreover, the platform allows precise adjustment of the ratio between different nucleic acid drugs based on the expression and interaction of target genes, enabling personalized dosing and maximizing synergistic therapeutic outcomes through optimized proportioning of multi-target drugs, thus overcoming the limitations of single-target or fixed-ratio treatment regimens in complex diseases. In the future, the platform could be further integrated with chemotherapeutic agents or immunomodulators to construct multifunctional combination therapies. Therefore, BSAD represents not only a therapeutic tool for ESCC but also a customizable, proportion-adjustable multi-target nucleic acid drug delivery platform with broad potential for expanding precision medicine applications.

While these findings are encouraging, several limitations of this study warrant attention. All experimental conclusions were derived from a single ESCC cell line (KYSE450) and its subcutaneous xenograft models, which may not fully capture the heterogeneity present in the patient population with ESCC. Furthermore, challenges such as the scalable production of DNAzymes *in vivo* and potential off-target effects still require systematic resolution. Future work will focus on validating the efficacy of BSAD in more representative models, optimizing its production and storage processes. These efforts will further strengthen the foundation for clinical translation and expand the application prospects of proportionally adjustable nucleic acid nano-hybrids in precision medicine.

**Electronic Supplementary Material:** Supplementary material (including the full experimental section covering reagents, materials, and methods, characterization data of the nanohybrids, relevant DNA sequence lists, and other supporting experimental data) is available in the online version of this article at <https://doi.org/10.26599/NR.2026.94908592>.

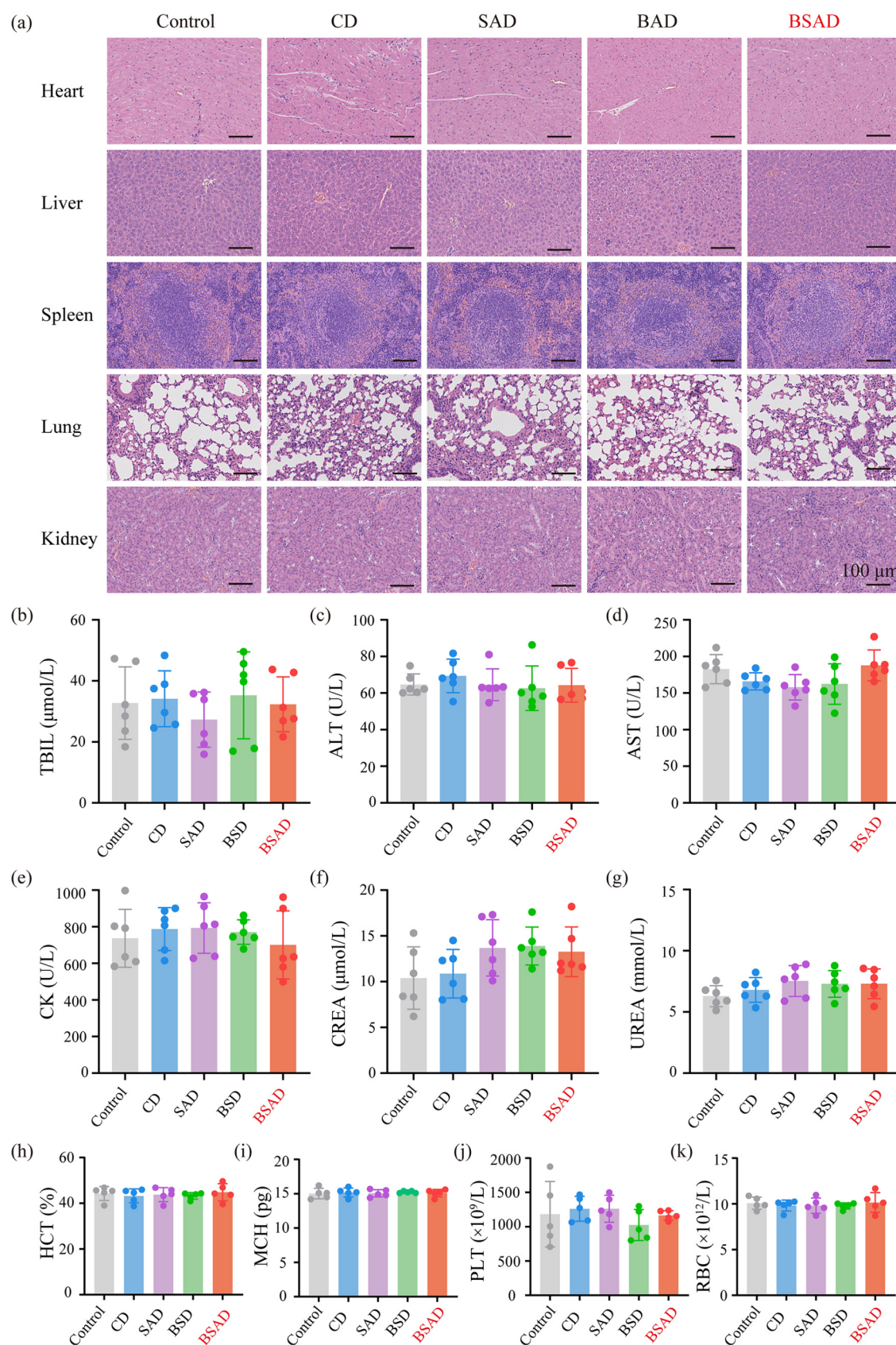


**Figure 5** *In vivo* biosafety of BSAD. (a) Representative H&E staining of major organs. Scale bars: 100 μm. Cardiac, hepatic, and renal function parameters ( $n = 6$ , in panels (b)–(g)), and hematological parameters ( $n = 5$ , data shown for tumor-bearing mice across treatment groups, in panels (h)–(k)). (b) TBIL, total bilirubin; (c) ALT, alanine aminotransferase; (d) AST, aspartate aminotransferase; (e) CK, creatine kinase; (f) CREA, creatinine; (g) UREA, urea; (h) HCT, hematocrit; (i) MCH, mean corpuscular hemoglobin; (j) PLT, platelet count; (k) RBC, red blood cell count.

## Data availability

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials.

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## Declaration of competing interest

The authors declare no competing interests.

## Author contribution statement

W. Y. Y., J. H. S., and J. J. S. designed the project. W. Y. Y., Z. G. D., W. L., J. H. S., and Y. T. L. performed the experiments and collected the data. All authors analyzed and interpreted the data, contributed to the writing of the manuscript, discussed the results and implications, and edited the manuscript at all stages.

## Informed consent

Not applicable.

## Ethics statement

All animal experiments were conducted in accordance with the approval of the Life Science Ethics Review Committee of Zhengzhou University (Certification Number: SCXK (Yu) 2023-0004). The ethical committee approval numbers for the animal experiment are 25-xyx-021 and 25-xyx-022.

## Use of AI statement

None.

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