

Nanozyme-mediated delivery of natural compounds induces ferroptosis to potentiate radiotherapy in bladder cancer

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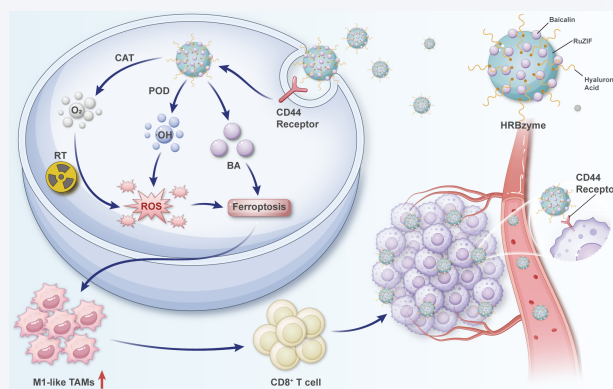
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ABSTRACT: Immune suppression within the tumor microenvironment (TME) is a major obstacle to effectively treating bladder cancer; however, inducing immunogenic cell death via ferroptosis has attracted increasing attention as a means to boost antitumor immunity. A ruthenium (Ru)-incorporated zeolitic imidazolate framework-8-based nanostructure loaded with the ferroptosis inducer baicalin (BA) and coated with a hyaluronic acid (HA) shell is described as a radioimmunotherapeutic platform for bladder cancer treatment. Incorporation of Ru, a high-atomic-number element, significantly increased local radiation energy deposition, thus improving radiotherapeutic outcomes at tumor sites. The synthesized nanozyme showed intracellular catalase-like activity, promoting oxygen production to suppress hypoxia and reduce radioresistance within the TME. Peroxidase-mimicking activity showed efficient generation of reactive oxygen species. HA functionalization demonstrated selective tumor homing by targeting the CD44 receptor, which is overexpressed in bladder cancer, enabling effective drug delivery to malignant tissue. BA was released *in situ*, reprogramming the immunosuppressive TME. When combined with ionizing radiation, this approach elicited a potent systemic immune response, effectively inhibiting primary tumor progression and metastasis. This strategy addresses immune suppression and provides a viable synergistic therapeutic approach for bladder cancer treatment.

KEYWORDS: nanozyme, radiotherapy, immunotherapy, hypoxia, bladder cancer



1 Introduction

Bladder cancer is among the most common malignancies of the urinary tract. It is clinically classified into non-muscle-invasive bladder cancer (NMIBC), muscle-invasive bladder cancer (MIBC), and metastatic disease [1, 2]. Although therapeutic options for these classifications are well established, their clinical efficacy remains

limited by poor outcomes, high rates of recurrence, and treatment-related morbidity, underscoring the pressing need for new combination therapeutic approaches [3]. For NMIBC, transurethral tumor resection followed by intravesical chemotherapy remains the standard of care, taking advantage of the bladder's hollow anatomy for localized drug delivery [4]. However, the limited permeability of urothelium and the potential of tumor cells to acquire chemoresistance frequently compromise treatment efficacy and lead to adverse effects. Therefore, NMIBC is associated with a 5-year recurrence rate of approximately 30%–70%, and therapeutic options after recurrence remain extremely limited. In MIBC, radical cystectomy combined with radiotherapy and chemotherapy can provide tumor control; however, this approach is highly invasive

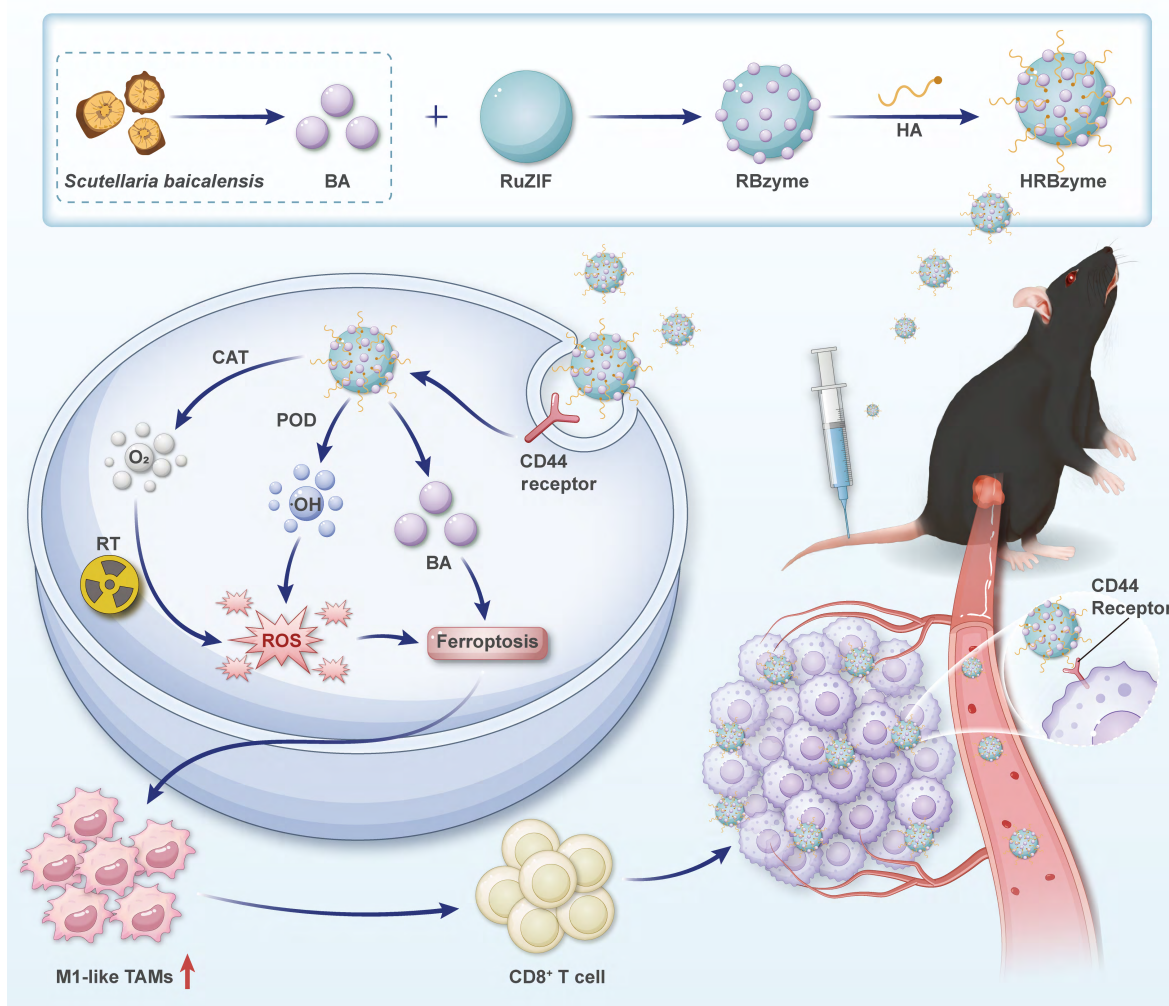
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and often results in significant impairment of urinary function and overall quality of life, while failing to fully prevent disease relapse or metastasis [5, 6]. Moreover, patients who are ineligible for surgery face limited treatment options. In this complex clinical setting, combined therapeutics that merge radiotherapy and immunotherapy have attracted considerable attention. Besides its direct cytotoxic effects, radiotherapy can trigger immunogenic cell death and boost anti-tumor immune responses, opening new possibilities for improving bladder cancer treatment [7]. However, the highly immunosuppressive characteristics of the bladder cancer tumor microenvironment (TME), along with the innate radioresistance of malignant cells, substantially diminish the therapeutic benefit of radiotherapy or immunotherapy when used as monotherapies. As a result, simultaneously improving the localized tumor-killing efficiency of radiotherapy while also systemically reversing TME-mediated immune suppression has emerged as an important challenge in advancing bladder cancer treatment [8–10]. In this case, developing advanced nanotherapeutic platforms that function as both radiosensitizers and immunomodulators, thus overcoming tumor resistance and immune evasion through multiple mechanisms, shows great promise for achieving effective, low-toxicity bladder cancer therapies [11, 12].

Traditional Chinese medicine (TCM) has historically played a key role in disease prevention and treatment, mainly due to its broad spectrum of bioactive components [13, 14]. Advances in modern biomedical research have further demonstrated that natural TCM-derived products have remarkable chemical diversity due to complex structures and numerous functional groups. By coordinating multiple components, molecular targets, and signaling pathways, these compounds can regulate physiological homeostasis [15, 16]. For example, different classes of TCM-derived bioactive compounds, such as flavonoids, terpenoids, and alkaloids, show specific pharmacological effects. Despite these advantages, the clinical translation of natural compounds into modern therapeutics remains challenging. Many TCM-derived active components are limited by unfavorable pharmacokinetic properties, i.e., poor solubility, poor *in vivo* stability, rapid metabolic clearance, and low systemic bioavailability [17–19]. These drawbacks lead to variable, often unpredictable therapeutic outcomes, restricting their broader application in advanced treatment modalities. It should be noted that baicalin (BA), a major bioactive component derived from the traditional Chinese medicine *Scutellaria baicalensis*, exhibits anti-inflammatory, antioxidant, and antitumor activities, and is capable of inducing ferroptosis [20–24]. Its mechanisms of action include inducing tumor cell apoptosis, suppressing cellular proliferation,



Scheme 1 Schematic representation of the HRBzyme synthesis and its radiosensitization-driven immunomodulatory mechanism, highlighting M1 polarization and its activation of antitumor immune responses.

and promoting macrophage polarization toward the pro-inflammatory M1 phenotype, thus inhibiting tumor growth and metastasis [25]. However, its systemic cytotoxicity cannot be overlooked. Poor water solubility and non-specific biodistribution may lead to off-target damage to normal tissues. Based on the property that BA induces ferroptosis through inhibiting glutathione peroxidase 4 (GPX4) and depleting glutathione (GSH), the rapid advancement of nanotechnology offers a promising strategy to address these limitations [26–28]. The rational design of nanoscale delivery platforms can enhance the stability, bioavailability, and *in vivo* performance of TCM-derived compounds, improving therapeutic efficacy and addressing the shortcomings of traditional formulations.

Nanozymes are artificially engineered nanomaterials with intrinsic enzyme-mimicking catalytic activity, capable of emulating the functions of endogenous enzymes, i.e., peroxidase (POD), catalase (CAT), and superoxide dismutase (SOD) [29–32]. Due to these properties, nanozymes have attracted increasing attention as versatile platforms for biomedical and oncological applications [33]. Previous work has demonstrated the successful development of bimetallic nanozyme systems that show dual POD- and glutathione oxidase (GSH-OXD)-like activities, effectively inducing excessive reactive oxygen species (ROS) accumulation and demonstrating synergistic photothermal therapeutic effects in bladder cancer [34–36]. The basic therapeutic mechanism of such nanozymes relies on the continuous amplification of oxidative stress within tumor cells via catalytic reactions. When ROS levels exceed the intrinsic antioxidant buffering capacity of cancer cells, redox homeostasis within the TME is disrupted, ultimately leading to tumor cell death [37–40]. Nanozyme catalytic properties, such as active sites and nanoscale structure, can be finely tuned through material design to improve catalytic efficiency for specific therapeutic goals. Based on these principles and utilizing the nanoscale features and high catalytic activity of nanozymes, this study introduces HRBzyme, a novel nanozyme-based delivery system. HRBzyme is designed to deliver BA, a bioactive compound from TCM, directly and efficiently to tumor tissue, while exploring its therapeutic potential in combination with radiotherapy and immunotherapy for bladder cancer (Scheme 1). Hyaluronic acid (HA), an anionic linear glycosaminoglycan composed of repeating disaccharide units of D-glucuronic acid and D-N-acetylglucosamine, has a strong affinity for the CD44 receptor, which is often overexpressed on the surface of bladder cancer cells. This interaction confers active tumor targeting. HRBzyme is designed with a Ru-doped zeolitic imidazolate framework material (Ru-ZIF) nanozyme core, loaded with BA, and coated with an HA layer to promote CD44-targeted delivery. After systemic administration, HRBzyme tends to accumulate in CD44-overexpressing bladder cancer cells, disrupt intratumoral redox homeostasis through enzyme-like catalytic activity, and release BA locally. When combined with ionizing radiation, this multifunctional system further enhances antitumor immune responses and directs macrophages toward the M1 inflammatory state, boosting tumor destruction and reducing metastasis.

2 Results and discussion

After successfully synthesizing the Ru-ZIF nanoplatform, BA was incorporated into the porous structure, followed by the addition of HA as an external coating layer. Transmission electron microscopy

(TEM) images in Fig. 1(a) show the morphology of HRzyme, while Fig. 1(b) displays that of HRBzyme. Both nanostructures have a uniform spherical shape with an average size of around 80 nm. HRBzyme features a visible thin surface layer, confirming the effectiveness of the HA coating. The colloidal stability of Ru-ZIF and HRBzyme was examined by monitoring particle size changes in phosphate-buffered saline (PBS). As shown in Fig. 1(c), no significant changes in hydrodynamic diameter were observed for either formulation over time, confirming their favorable stability under physiological conditions. Moreover, the diameter distribution and zeta potential of Ru-ZIF and HRBzyme were demonstrated in Figs. S1 and S2 in the Electronic Supplementary Material (ESM), respectively. Elemental mapping of HRBzyme in Fig. 1(d) verified the existence of Zn, O, Ru, N and C elements in HRBzyme. The X-ray diffraction (XRD) pattern is demonstrated in Fig. 1(e). X-ray photoelectron spectroscopy (XPS) was used to analyze the surface chemical composition and valence states of elements in HRBzyme. The XPS survey spectrum indicated that HRBzyme is composed mainly of Ru, C, and N (Fig. 1(f)). High-resolution XPS spectra of Ru³⁺ revealed two characteristic binding energy peaks at approximately 475.86 and 462.28 eV, corresponding to the Ru 3p orbitals (Fig. S3 in the ESM). The release of BA from HRBzyme under different pH conditions was also examined. As shown in Fig. 1(g), BA release was significantly higher in acidic conditions, with over 40% released within 25 min at pH 6.5. The loading efficiency of BA and HA ligands was calculated to be 2.37% according to the absorption curve (Fig. S4 in the ESM). These results confirm the successful construction of HRBzyme and its ability to rapidly release BA in the acidic TME.

Cellular uptake was observed by co-incubation of MB49 cells with RBzyme and HRBzyme, respectively. As shown in Fig. S5 in the ESM, higher green fluorescence intensity representing HRBzyme was observed, indicating higher cellular uptake of HRBzyme by MB49. Given the crucial role of ROS as secondary messengers in M1 macrophage signaling pathways, which are vital for initiating and maintaining the M1-like phenotype, the ROS-generating capacity of HRBzyme was evaluated. Its CAT- and POD-like activities were tested using standard protocols [41]. As illustrated in Fig. 2(a), rapid oxygen release was observed when HRBzyme was exposed to H₂O₂, reaching a concentration of 17.6 mg/L within 10 min. The POD-like activity of HRBzyme was examined using tetramethylbenzidine (TMB) as a chromogenic substrate. In the presence of hydroxyl radicals (·OH), colorless TMB was oxidized to a blue product. The absorbance at 652 nm was measured for HRBzyme-treated TMB in the presence of H₂O₂, with varying concentrations of TMB and H₂O₂, as shown in Fig. 2(b). Kinetic analysis using TMB as the substrate revealed that HRBzyme followed Michaelis–Menten kinetics, with a maximum reaction velocity ($V_{\max}$) of 2.28×10^{-7} M/s and a Michaelis constant (K_m) of 7.72 mM (Figs. 2(c) and 2(d)). The coexistence of CAT- and POD-mimicking activities endows HRBzyme with the dual capability to enhance oxidative stress while simultaneously alleviating hypoxia.

Ferroptosis is a regulated, non-apoptotic form of cell death characterized by iron-dependent lipid peroxidation and plays a critical role in tumor cell elimination. Given that excessive ROS accumulation can disrupt intracellular redox homeostasis, promote oxidative stress, and lead to lipid peroxidation, ultimately inducing ferroptosis, the ability of HRBzyme to eradicate tumor cells via ferroptosis was further explored. TEM images of MB49 cells treated with HRBzyme showed significant ultrastructural changes,

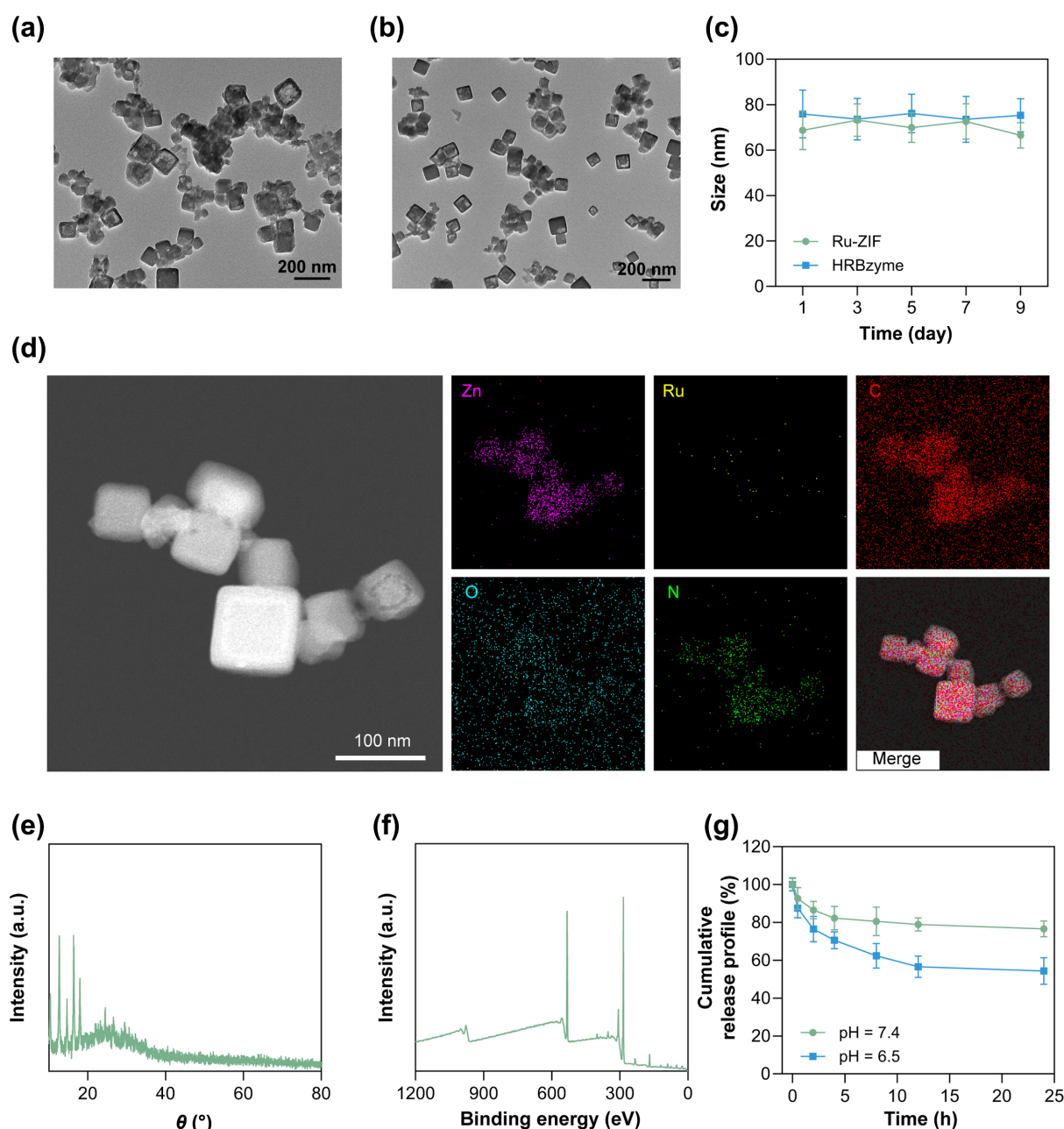


Figure 1 Synthesis and characterization of HRBzyme. (a) TEM images of HRzyme and (b) HRBzyme. (c) Size change of HRzyme and HRBzyme. (d) Elemental mapping of HRBzyme. (e) XRD pattern and (f) XPS spectra of HRBzyme. (g) Release profile of BA. Mean $\pm$ SD are shown for all datasets, $n = 3$.

including a significant reduction in mitochondrial cristae (Fig. 3(a)). To further validate ferroptosis induction, the expression of GPX4, a key enzyme that inhibits lipid peroxidation, along with the level of 4-hydroxynonenal (4-HNE), a terminal byproduct of lipid peroxidation, was assessed using western blotting after 12 h of treatment (Fig. 3(b)). The HRBzyme combined with radiotherapy (RT) (HRBzyme+RT) group showed a significant decrease in GPX4 levels and an increase in 4-HNE, indicating severe redox disruption and activation of ferroptosis. Immunofluorescence with boron-dipyrromethene (BODIPY) probes and confocal laser scanning microscopy (CLSM) revealed the highest lipid peroxide (LPO) accumulation in this group (Fig. 3(c)). This group also showed the greatest depletion of intracellular GSH among all treatment conditions (Fig. 3(g)). Malondialdehyde (MDA), a marker of lipid

peroxidation-induced damage, was highest in the HRBzyme+RT group (Fig. 3(h)). These results confirm the induction of ferroptosis by HRBzyme+RT. The ability of HRBzyme to mitigate hypoxia was evaluated by analyzing hypoxia-inducible factor 1- α (HIF-1 α) protein levels via western blotting. As shown in Fig. S6 in the ESM, HRBzyme treatment significantly reduced HIF-1 α expression compared to the control group, indicating its efficacy in alleviating hypoxic stress. Intracellular ROS production under different treatment conditions was quantified using the 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) probe. As shown in Fig. 3(d), minimal green fluorescence was detected in the RT group alone, indicating limited ROS production. In comparison, HRBzyme treatment significantly increased ROS levels due to its POD-like catalytic activity. The most significant ROS accumulation

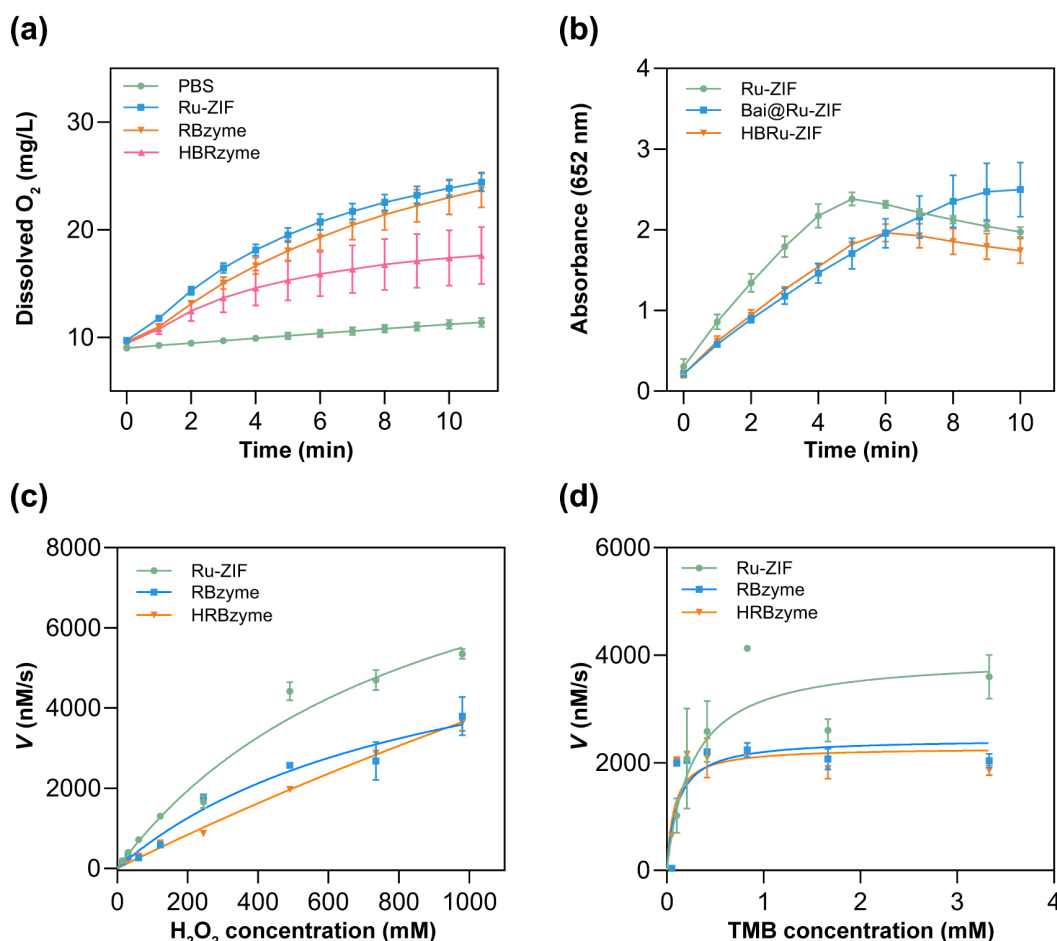


Figure 2 Dual POD and CAT-like activity. (a) Generation of dissolved O₂. (b) Absorbance at 652 nm of TMB in the presence of H₂O₂. Absorbance at 652 nm in relation to (c) TMB concentration and (d) H₂O₂ concentration, respectively. Mean $\pm$ SD are shown for all datasets, $n = 3$.

was observed in the HRBzyme+RT group, with fluorescence intensity exceeding that of the HRzyme+RT, HRBzyme, and RT groups by factors of 1.65, 3.68, and 8.41, respectively. Although ionizing radiation alone induced moderate DNA damage, the HRBzyme+RT group showed the strongest red fluorescence signals, consistent with quantitative fluorescence analysis (Fig. 3(e)). Cell viability was further assessed using live/dead staining and quantitative assays (Fig. 3(f)). Live/dead analysis revealed 29.4% cell death in the HRzyme+RT group, whereas $> 50\%$ of cells were nonviable in the HRBzyme+RT group. Consistent with these findings, a cell counting kit-8 (CCK-8) assay showed a reduction in cell viability to 56.6% in the HRBzyme+RT group, corresponding to only 0.69-fold the viability observed in the RT group, confirming the increased cytotoxicity of HRBzyme+RT (Fig. 3(i)). Long-term effects on cellular proliferation were further examined using colony formation assays (Fig. 3(j)), which indicated substantial radiosensitization and effective suppression of tumor cell proliferation by HRBzyme.

Dendritic cells (DCs) are widely recognized as the most efficient antigen-presenting cells and serve as a pivotal bridge between innate and adaptive immune responses. Their dysfunction or poor activation is closely related to tumor immune escape and the establishment of immune tolerance. In their immature state, DCs have strong migratory capacity and are highly efficient at antigen uptake, intracellular processing into peptide fragments, and migration to draining lymph nodes. After maturation within

lymphoid tissues, DCs acquire the ability to activate naïve T lymphocytes, thus initiating antigen-specific adaptive immune responses. In this study, the ability of HRBzyme+RT to promote DC maturation was evaluated. In the transwell coculture experiment, treated MB49 tumor cells were seeded in the upper chamber, while bone marrow-derived dendritic cells (BMDCs) were cultured in the lower chamber. Figures 4(a) and 4(b) show immunofluorescence images of calreticulin (CRT) exposure on MB49 cells. The HRzyme+RT group showed increased CRT signals, and the HRBzyme+RT group had higher fluorescence intensity. Quantitative analysis indicated that CRT-positive cells in the HRBzyme+RT group were 1.5 times more than those in the HRzyme+RT group. Next, high mobility group box 1 (HMGB1) release, a key marker of immunogenic cell death, was examined. As shown in Fig. 4(c), the HRBzyme+RT group showed a significant decrease in nuclear HMGB1 and the highest levels of extracellular HMGB1 (Fig. 4(d)). The expression of the costimulatory molecules (CD80 and CD86) on DCs was also examined in this study. Flow cytometry (FCM) (Fig. 4(e), and Figs. S7 and S8 in the ESM) showed that HRBzyme+RT treatment significantly upregulated CD80 and CD86 expression, indicating effective DC maturation mediated by HRBzyme-enhanced radiosensitization.

Besides their role in tumor infiltration, M1-polarized macrophages are crucial for antitumor immunity through direct phagocytosis of tumor cells, antigen presentation to T lymphocytes, and reprogramming of immunosuppressive M2 macrophages by

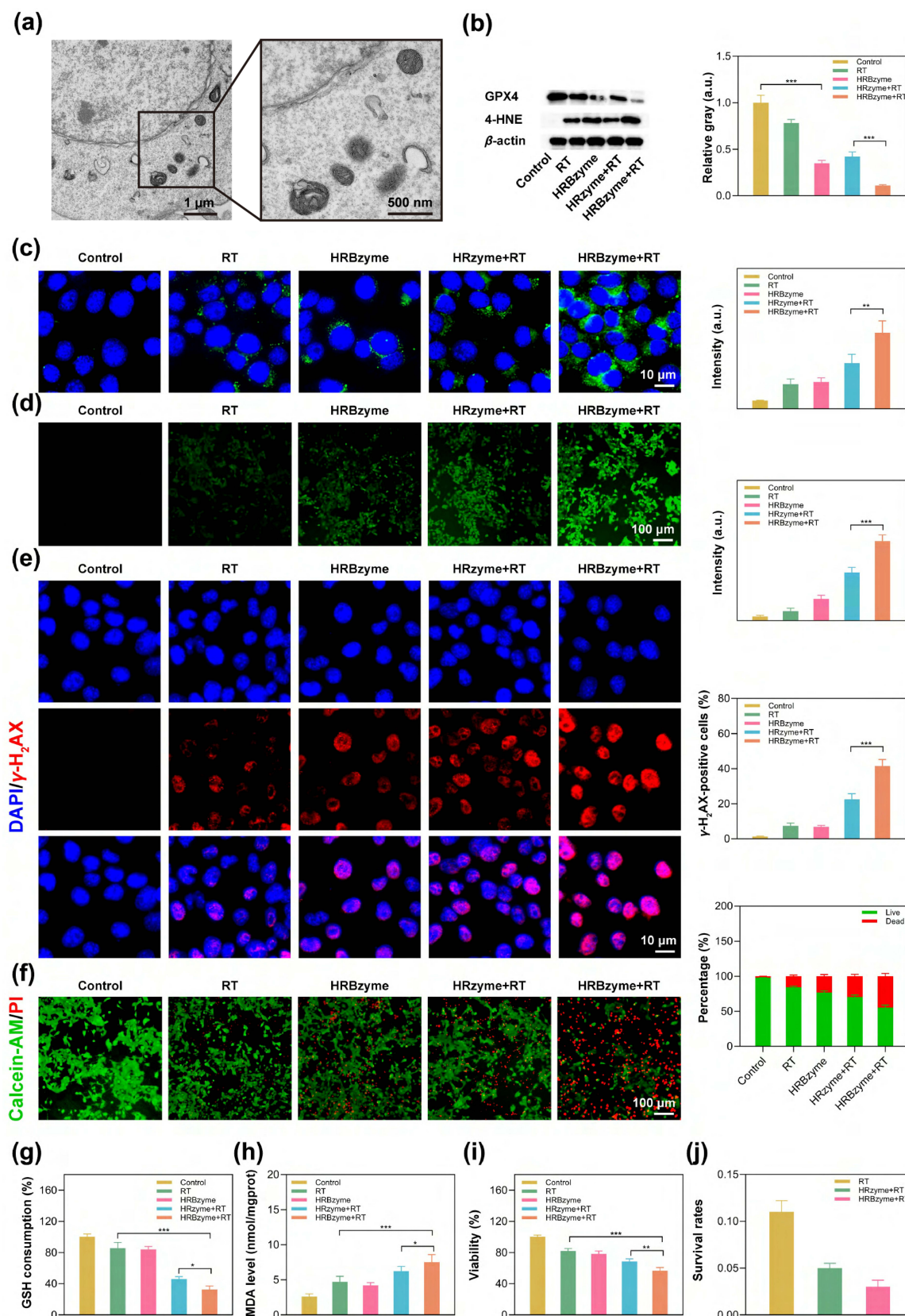


Figure 3 *In vitro* therapeutic effect of HRBzyme-enhanced RT. (a) TEM images of MB49 cells co-incubated with HRBzyme. (b) Western blotting analysis of GPX4 and 4-HNE expression. (c) Representative CLSM images of MB49 cells under various treatments to assess LPO accumulation and fluorescence intensity. (d) ROS generation was evaluated using DCFH-DA and quantified by fluorescence intensity. (e) DNA damage was examined using γ -H₂AX staining and quantified by fluorescence intensity. (f) Live/dead cell assay and quantification analysis. (g) GSH level. (h) MDA level. (i) Viability of MB49 cells in different treatment groups. (j) Cell colony assay. Mean $\pm$ SD are shown for all datasets, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, One-way ANOVA, $n = 3$.

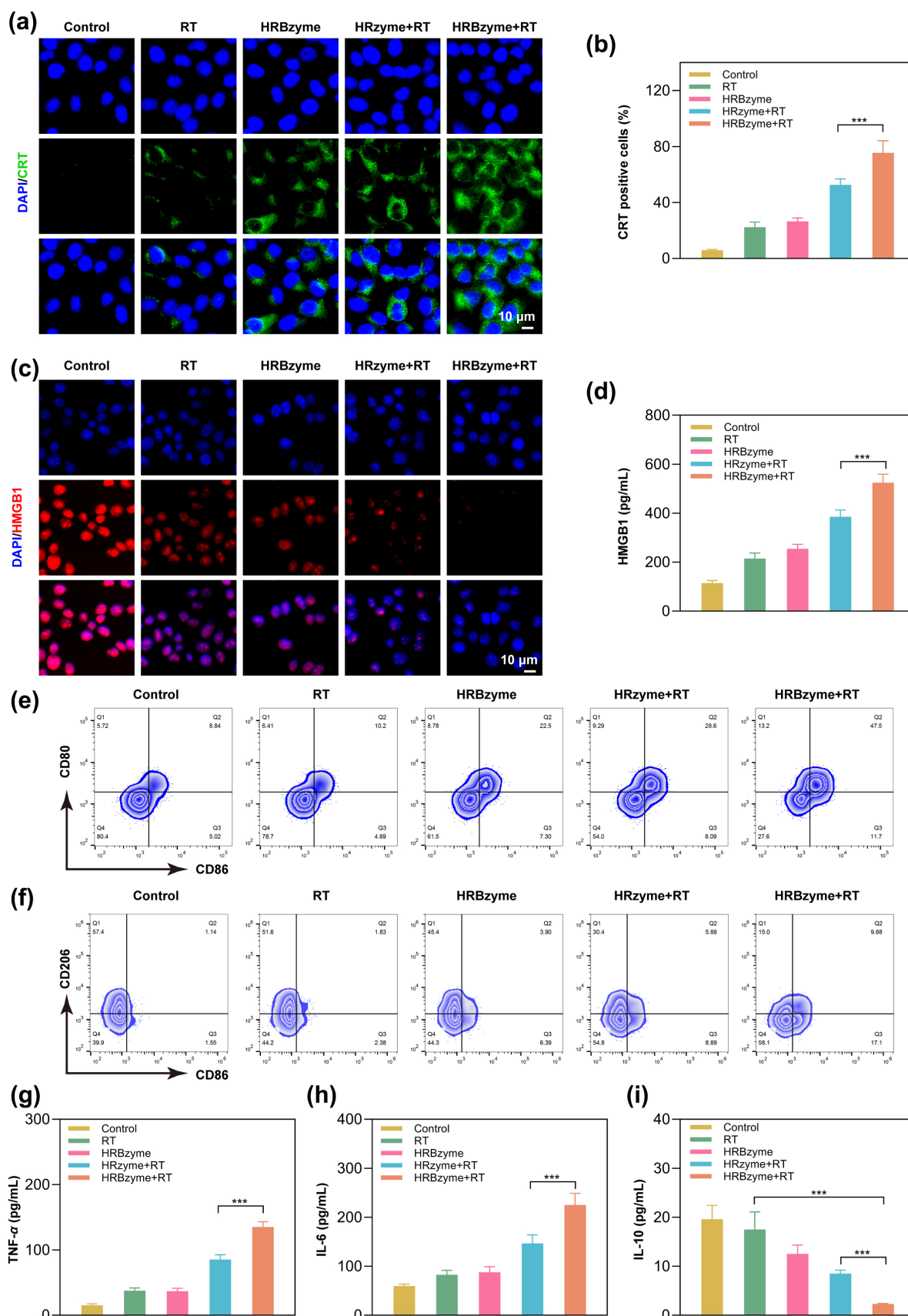


Figure 4 HRBzyme improved RT to evoke the ICD effect. (a) Immunofluorescent images of CRT. (b) Statistical analysis of CRT-positive cells. (c) Immunofluorescent images of HMGB1. (d) Measurements of released HMGB1. (e) Matured DCs. (f) M1-like BMDMs polarization. (g) TNF- α , (h) IL-6 secretion, and (i) IL-10 level. Mean $\pm$ SD are shown for all datasets, *** $P < 0.001$, One-way ANOVA, $n = 3$.

secreting pro-inflammatory cytokines and chemokines. The nanozyme platform described in this study was engineered to promote macrophage M1 polarization and immune activation by enhancing ROS and regulating BA release. Therefore, the capacity of HRBzyme+RT to drive macrophage polarization toward the M1 phenotype was investigated *in vitro*. The polarization effects of various treatments were evaluated using interleukin-4 (IL-4)-induced M2-polarized BMDMs. Flow cytometry revealed a slight increase in M1-like macrophages (CD206⁺CD86⁺) in the RT and HRBzyme groups, reaching 2.38% and 6.39%, respectively (Fig. 4(f), and Figs. S9 and S10 in the ESM). The combination of HRzyme+RT further promoted M2-to-M1 repolarization, due to the radiosensitizing effect of HRzyme. The HRBzyme+RT group showed the most significant shift toward the M1 phenotype, with 17.1% M1-like macrophages, about 1.92 times higher than in the HRzyme+RT group. Enzyme-linked immunosorbent assay (ELISA) analysis of cytokine secretion further confirmed these results. Under irradiation conditions, HRBzyme significantly elevated levels of M1-associated pro-inflammatory cytokines, i.e., tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6), compared with control treatments (Figs. 4(g)–4(i)), consistent with flow cytometry data. However, interleukin-10 (IL-10) secretion, an anti-inflammatory cytokine, was reduced to 13.1% in the HRBzyme+RT group compared with the RT group. These findings indicate that HRBzyme combined with radiotherapy effectively triggers immunogenic cell death, enhances DC maturation, and reprograms macrophages from an M2 to an M1 phenotype, causing a robust antitumor immune response.

Before examining the tumor-targeting ability and intratumoral accumulation of HRBzyme, this study analyzed its biosafety profile. Hemolysis rate assessment in Fig. S11 in the ESM verified the satisfactory biocompatibility of HRBzyme. Next, healthy mice were administered PBS or HRBzyme. Their body weights were monitored throughout the observation period, and at the study endpoint, blood samples and major organs were collected. Histological examination of hematoxylin and eosin (H&E)-stained sections from the heart, liver, spleen, lungs, and kidneys showed that, compared to saline controls, mice treated with HRBzyme maintained normal tissue architecture. No significant pathological changes (tissue necrosis, inflammation, fibrosis, or structural damage) were observed in any examined organs (Fig. 5(a)). Routine blood analysis confirmed that key parameters, including white blood cell (WBC), red blood cell (RBC), and platelet (PLT) counts, in mice treated with HRBzyme remained within normal ranges, with no signs of bone marrow suppression or systemic infection. Serum biochemical tests for liver and kidney function, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), and blood urea nitrogen (BUN), showed no statistically significant increases compared to the control group, indicating no apparent liver or kidney damage (Figs. 5(c) and 5(d)). During the dosing period, all animals maintained normal behavior, activity levels, and feeding patterns. Consistent with these observations, longitudinal body weight measurements also showed similar growth patterns between the treated and control groups, with no evidence of weight loss or growth retardation (Fig. 5(b)).

To assess *in vivo* biodistribution and tumor accumulation, two Cy3-labeled nanoprobe, Cy3-labeled RBzyme and Cy3-labeled HRBzyme, were intravenously administered to mice bearing subcutaneous tumors. After 24 h, major organs (heart, liver, spleen, lungs, and kidneys) and tumor tissues were harvested for *ex vivo*

fluorescence imaging. The imaging results showed a stronger fluorescence signal at the tumor site in mice treated with HRBzyme compared with RBzyme, with approximately 3.07 times the signal intensity, indicating enhanced tumor localization. To further confirm these findings, the mean fluorescence intensity (MFI) was calculated to quantitatively analyze distribution across tissues. As shown in Figs. 5(e) and 5(f), the strong targeting ability of HRBzyme is probably due to HA on its surface, while lower liver and renal accumulation was discovered in HRBzyme. *In vivo* fluorescence imaging also confirmed that more apparent fluorescence signals were observed in the HRBzyme group (Fig. S12 in the ESM). HA selectively binds to the CD44 receptor, which is abundantly expressed on tumor cells, promoting active targeting in tumor tissue. These findings confirm HRBzyme's biosafety profile and its effective ability to target tumors *in vivo*.

Based on the above findings, an MB49 mouse bladder cancer model was developed to further evaluate the therapeutic efficacy of HRBzyme RT *in vivo*. Tumor-bearing mice were randomly divided into five groups: (1) control, (2) RT alone, (3) HRBzyme alone, (4) HRzyme+RT, and (5) HRBzyme+RT. As shown in the tumor growth curves in Fig. 6(a), rapid, uncontrolled tumor progression occurred in the control, RT, and HRBzyme monotherapy groups. In comparison, the HRzyme+RT group showed significant tumor suppression, likely due to the combined catalytic and radiosensitizing effects of HRzyme. Upon addition of BA, HRBzyme demonstrated the most potent antitumor activity. Figures 6(b) and 6(d) reveal that tumors in the RT group reached approximately 600 mm³ by day 15, while those in the HRBzyme+RT group remained considerably smaller. These results were consistent with the tumor weight in Fig. S13 in the ESM. The body weight variation during observation did not exhibit significant differences among groups (Fig. 6(c)). The Kaplan-Meier survival curve in Fig. 6(e) confirmed that combining HRBzyme with RT significantly extended the survival of tumor-bearing mice. Throughout treatment, body weights in all groups remained stable, indicating a good safety profile for these therapies.

Histological and molecular analyses of the excised tumor tissues provided mechanistic insights into the enhanced antitumor effects of HRBzyme combined with RT. H&E staining (Fig. 6(f)) showed minimal necrosis in control tumors. In comparison, both the RT-only and HRBzyme-only groups showed moderate tumor suppression with localized necrotic areas. The extent of tumor necrosis was significantly greater in the HRzyme+RT group, with the HRBzyme+RT treatment causing the most extensive necrosis throughout the tumor tissue. Immunohistochemical analysis further demonstrated significant alterations in tumor proliferation and cell death markers. Ki67 expression was reduced in the HRBzyme+RT group, indicating effective inhibition of cell proliferation, while terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining showed a substantial increase in apoptotic cells. Quantitative analysis showed that the TUNEL-positive area in the HRBzyme+RT group was 6.28 times larger than in the RT group alone (Figs. 6(g)–6(i)). HRBzyme+RT treatment resulted in LPO accumulation, as evidenced by a significant increase in red fluorescence intensity and downregulation of the ferroptosis-associated protein GPX4 (Figs. 6(j) and 6(k)). These results indicate that HRBzyme significantly enhances the therapeutic efficacy of RT by promoting LPO accumulation and synergistically inducing ferroptosis, which likely constitutes a key mechanism underlying its potent antitumor activity *in vivo*.

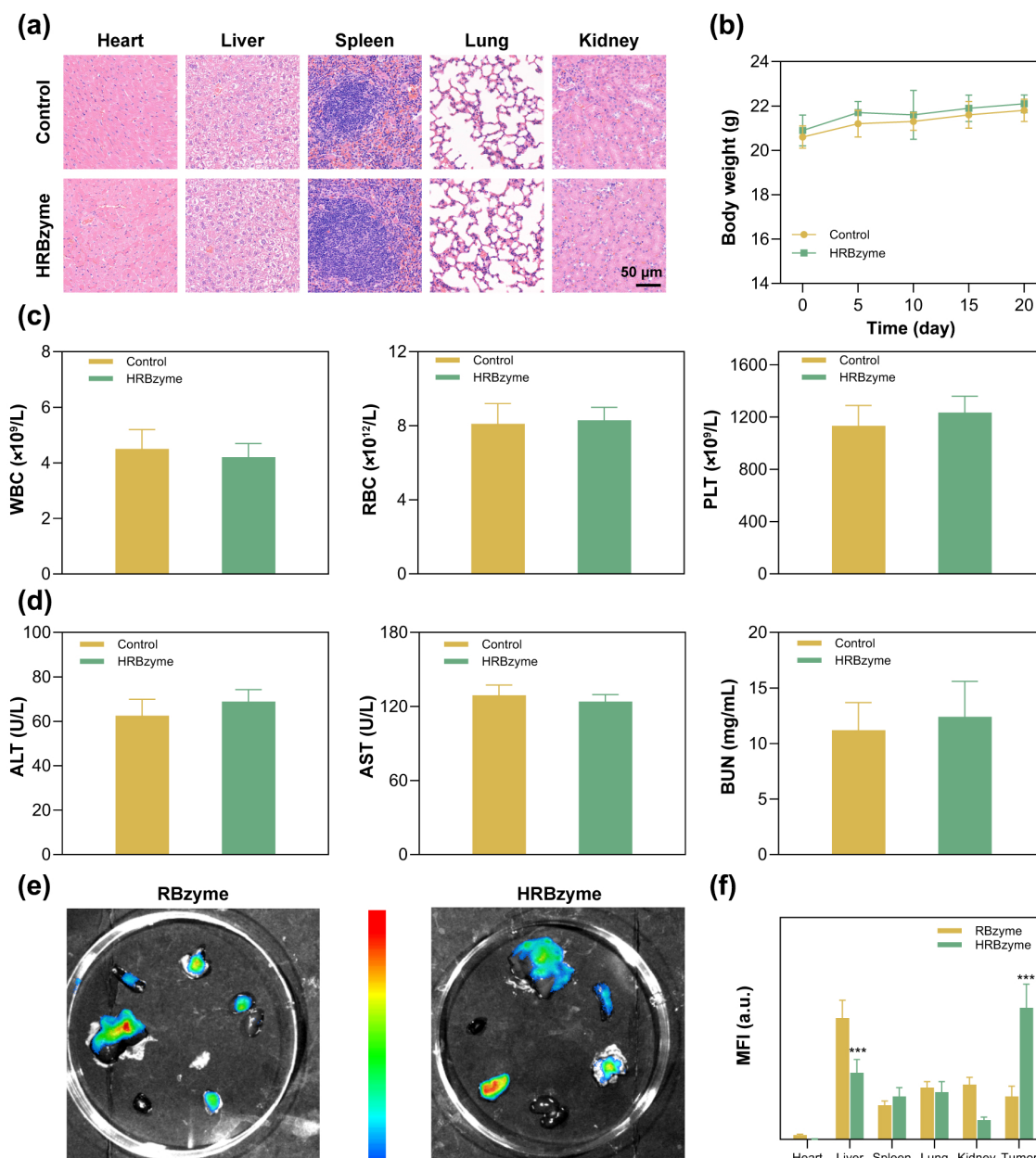


Figure 5 Biosafety evaluation and tumor accumulation assessment. (a) H&E staining of main organs. (b) Body weight monitoring. (c) Hematology analysis. (d) Biochemistry analysis in serum. (e) Ex vivo fluorescent imaging of mice administered with RBzyme and HRBzyme, respectively. (f) MFI in main organs and tumors at various time points after HRBzyme administration. Mean $\pm$ SD are shown for all datasets, *** $P < 0.001$, student's t-test, $n = 5$.

The study further explored the therapeutic efficacy of HRBzyme-mediated RT in more aggressive, metastatic tumor models. In a bilateral MB49 bladder cancer model using C57BL/6J mice, tumors were implanted on both flanks. Analysis of tumor growth kinetics revealed that the HRBzyme+RT group not only suppressed the progression of irradiated primary tumors but also inhibited growth and reduced the mass of contralateral tumors that did not receive direct irradiation (Figs. 7(a) and 7(d)). To elucidate the immunological mechanisms behind this phenomenon, macrophage polarization in tumor tissues was examined using immunofluorescence staining for inducible nitric oxide synthase (iNOS), a marker of M1 macrophages, and CD206, a marker of M2 macrophages. A significant reduction in CD206-positive macrophages was observed after HRBzyme+RT treatment, along

with an increase in iNOS-associated red fluorescence signals (Fig. 7(b)). Flow cytometry further confirmed a significant increase in the proportion of CD86-positive M1 macrophages (Fig. 7(e), and Figs. S14 and S15 in the ESM). Higher infiltration of cytotoxic CD8⁺ T cells was observed within the TME (Fig. 7(c)), together with higher levels of TNF- α and interleukin-6 (IL-6) (Figs. 7(f) and 7(g)). These results show that HRBzyme+RT effectively initiates a systemic antitumor immune response by reprogramming M2 macrophages into the M1 phenotype.

Considering lung metastasis is one of the most clinically relevant and life-threatening forms of distant tumor dissemination, a lung metastasis model was used. Considering the widespread overexpression of the CD44 receptor across multiple tumor cell lines (MB49, CT26, and 4T1), HRBzyme functionalized with HA

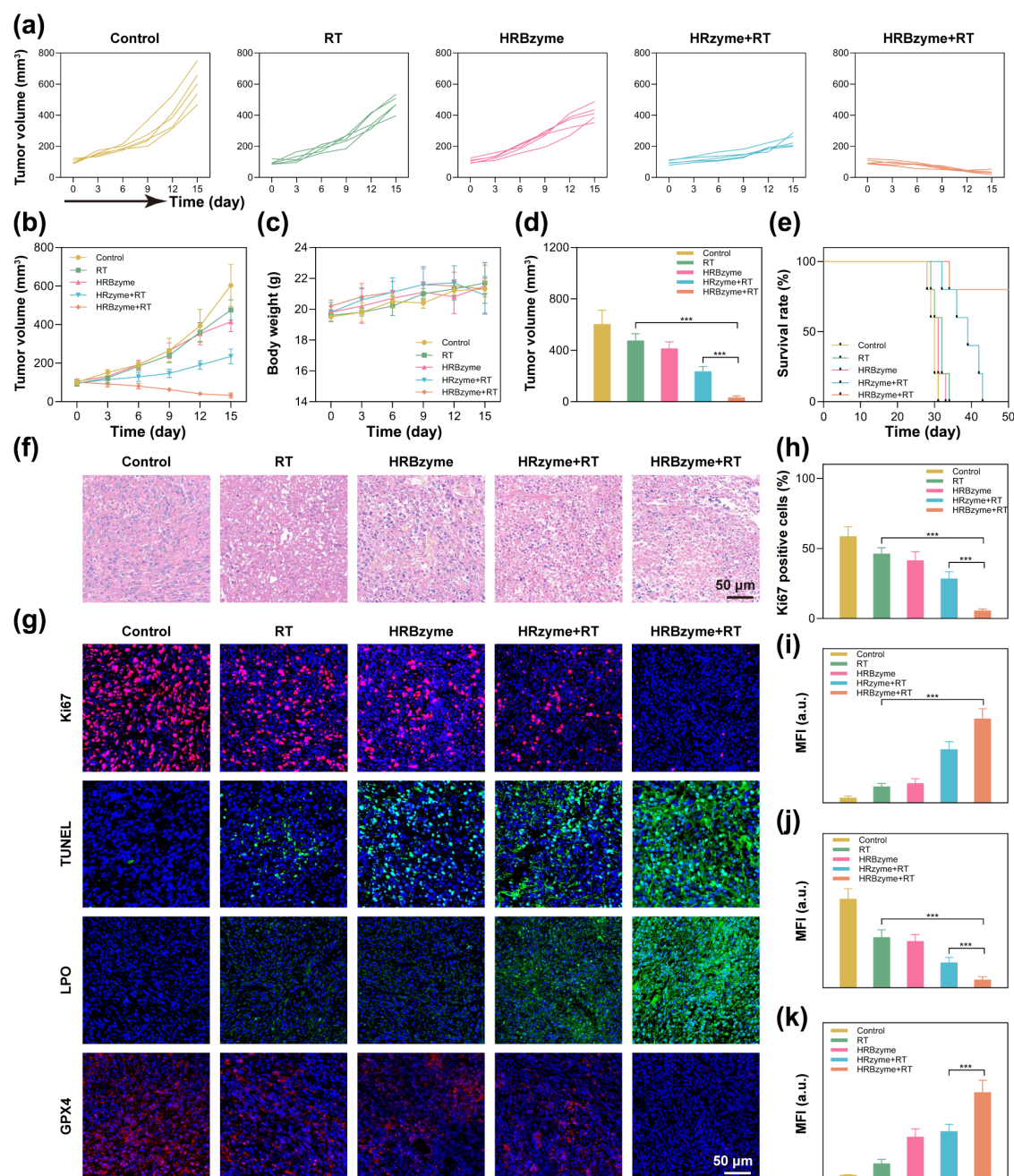


Figure 6 Antitumor activity of HRBzyme improved RT in MB49 tumor-bearing mice. (a) Individual tumor growth curves. (b) Tumor growth curves. (c) Body weight. (d) Tumor volume at day 18. (e) Survival curves of mice. (f) H&E staining of tumor tissues. (g) Immunofluorescent staining of Ki67, TUNEL, LPO, and GPX4. (h) Ki67-positive cells. (i) TUNEL positive area. Quantification of immunofluorescence intensity of (j) LPO and (k) GPX4. Mean $\pm$ SD are shown for all datasets, $^*P < 0.05$, $^{***}P < 0.001$, one-way ANOVA, $n = 5$.

was expected to target 4T1 cells actively. Therefore, a lung metastasis model was developed in BALB/c mice using the murine breast cancer cell line 4T1. As shown in Fig. 7(h), mice treated with HRBzyme-based combination therapy showed a significant decrease in metastatic lung nodules. Histopathological analysis also indicated reduced lung tissue damage in this group (Fig. 7(i)). Similarly, survival analysis revealed a significant extension in lifespan for mice receiving HRBzyme-based combination therapy (Fig. 7(j)). These results strongly suggest, compared with other previously reported ferroptosis-triggered nanozymes, that HRBzyme-enhanced RT elicits a potent systemic immune response

that can identify and eliminate circulating tumor cells, effectively preventing metastasis to distant organs [42–44].

3 Conclusions

This study reports the development of HRBzyme, a nanozyme that targets tumors, induces ferroptosis, and reprograms macrophages toward the M1 phenotype for bladder cancer treatment, significantly reducing metastasis. Its POD-like activity produces high levels of oxidative stress inside tumors. The HA outer shell allows HRBzyme to specifically bind to CD44 receptors, which are overexpressed on tumor cells, thus mediating active tumor targeting

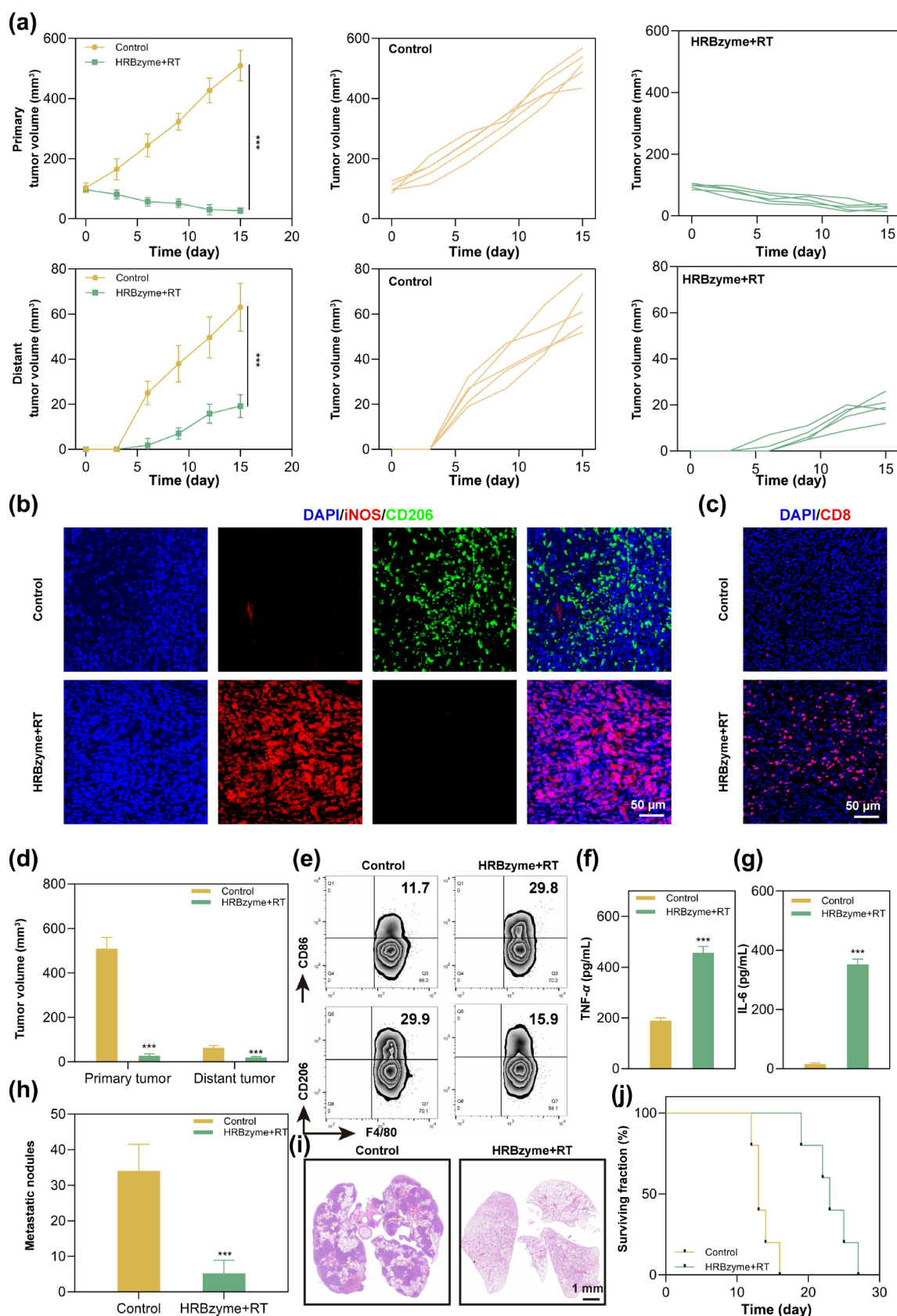


Figure 7 Reprogramming M2-to-M1 repolarization in metastasis models. (a) Individual primary and abscopal tumor growth curves across groups. (b) Representative immunofluorescence images for CD206 (green) and iNOS (red). (c) Representative immunofluorescence images for CD8. (d) Tumor volume at day 15. (e) FCM analysis of the expression of M1 markers (CD86⁺). Cytokine levels of (f) TNF-α and (g) IL-6. (h) Metastatic nodules. (i) H&E staining of the lung. (j) Survival curves. Mean ± SD are shown for all datasets, ***P < 0.001, student's t-test, n = 5.

and accumulation in the TME. Tumor hypoxia, a key factor in resistance to RT, was addressed by the CAT-like activity of HRBzyme, which catalyzes the decomposition of H_2O_2 into O_2 , boosting oxygen levels within the tumor, reducing hypoxia, and improving radiosensitivity. The incorporation of Ru enhances radiation absorption, thus increasing the delivered radiation dose during treatment. BA, a plant flavonoid derived from *Scutellaria baicalensis*, synergizes with nanozyme-induced oxidative stress to reprogram tumor-associated macrophages into an M1 phenotype, boosting antitumor immune responses. Experimental results show that HRBzyme improves radiotherapy outcomes, triggers ferroptosis in tumor cells, and inhibits tumor growth and metastasis. This study introduces a nanozyme delivery system based on natural products for bladder cancer treatment, presenting a promising approach to improve radioimmunotherapy by combining tumor targeting, radiosensitization, ferroptosis induction, and modulation of the TME.

Electronic Supplementary Material: Supplementary material (DLS measurements, zeta potential measurements, high-resolution XPS, absorption curve, cellular uptake, Western blot analysis of HIF-1 α , gating strategy, DC proportion, CD206-CD86⁺ cell proportion, hemolysis rate measurements, *in vivo* fluorescence imaging, tumor volume, F4/80-CD86⁺ cell proportion and F4/80-CD206⁺ cell proportion) is available in the online version of this article at <https://doi.org/10.26599/NR.2026.94908904>.

Data availability

Additional data related to this paper may be requested from the corresponding author upon request.

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Declaration of competing interest

All the contributing authors report no conflict of interests in this work.

Author contribution statement

L. Y. L.: Data curation, project administration, writing manuscript, experimental design. X. F. F.: Project administration, supervision, validation. M. L.: Investigation, writing manuscript. W. J.: Investigation, writing manuscript. E. J.: Project administration, validation, supervision. K. L. F.: Project administration, funding acquisition, supervision. Z. Y. Z.: Project administration, funding acquisition, supervision. All the authors have approved the final manuscript.

Informed consent

Not applicable.

Ethics statement

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Jilin University (Ethics code: 20260119-02).

Use of AI statement

None.

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