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Nanobody-Conjugated Theranostic Prodrug Targeting $\alpha_v\beta_3$ Integrin Enables Precision Cancer Therapy With Real-Time Imaging

Sanu Karan¹ | Hyunseung Lee¹ | Rema Naskar² | Mi Young Cho¹ | Hye Sun Park¹ | Eun Hee Han¹ | Soumyadip Hore³ | Sun Hee Cho¹ | Truc Ngoc Kim Lam^{1,4} | Taewoong Son^{1,4} | Tae Jung Park^{1,5} | Inkyu Hwang² | Jonathan L. Sessler⁶  | Kwan Soo Hong^{1,4,5} 

¹Center For Target-to-Therapeutics Research, Korea Basic Science Institute, Cheongju, Republic of Korea | ²Immunology and Immunopharmacology Laboratory, College of Pharmacy, Chungnam National University, Daejeon, Republic of Korea | ³Department of Chemistry, Korea Advance Institute of Science and Technology, Daejeon, Republic of Korea | ⁴Graduate School of Analytical Science and Technology, Chungnam National University, Daejeon, Republic of Korea | ⁵Department of Chemistry, Chung-Ang University, Seoul, Republic of Korea | ⁶Department of Chemistry, The University of Texas at Austin, Austin, Texas, USA

Correspondence: Jonathan L. Sessler (sessler@cm.utexas.edu) | Kwan Soo Hong (kshong@kbsi.re.kr)

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ABSTRACT

Theranostic prodrugs that integrate diagnostic and therapeutic functionalities represent a promising avenue for precision oncology. In this study, we report the rational design, synthesis, and preclinical evaluation of a nanobody-based $\alpha_v\beta_3$ integrin-targeted theranostic prodrug (**NBD**). The system incorporates a glutathione-responsive disulfide linker to enable selective release of doxorubicin within the tumor microenvironment, alongside an aza-BODIPY near-infrared fluorophore that provides activatable imaging capability. Compared to its cRGD peptide-conjugated counterpart (**cRBD**), **NBD** demonstrated superior tumor selectivity, intracellular activation, and cytotoxic potency in $\alpha_v\beta_3$ -overexpressing cancer cell lines. In vivo studies using SKOV3 xenograft models confirmed prolonged tumor retention, enhanced drug accumulation, and potent tumor suppression, with minimal systemic toxicity. Moreover, real-time imaging enabled precise monitoring of prodrug activation and distribution. These findings highlight the unique advantages of nanobody-guided theranostic platforms in enabling targeted drug delivery, reducing off-target effects, and facilitating image-guided therapy. The modular design of **NBD** provides a versatile framework for advancing personalized cancer treatment strategies.

1 | Introduction

Precision medicine and theranostics are approaches to healthcare that offer the promise of tailoring treatments and diagnostics to individual patient characteristics, thereby improving outcomes, reducing side effects, and increasing diagnostic accuracy [1, 2]. Precision medicine involves customizing therapies that exploit

disease-specific information based on genetic, molecular, and environmental factors. It is exemplified by targeted treatments, such as imatinib for chronic myeloid leukemia, which specifically inhibits the BCR-ABL fusion protein [3, 4]. In favorable cases, the precision medicine approach accelerates drug development through the identification of biomarkers, reduces clinical trial failures by targeting appropriate patient groups, and lowers

Sanu Karan, Hyunseung Lee, and Rema Naskar contributed equally to this work.

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healthcare costs [5, 6]. In contrast, theranostics integrate therapeutic and diagnostic tools into one construct. Ideally, this combination enables the real-time monitoring of treatment efficacy and disease progression, as embodied in radiolabeled monoclonal antibodies for imaging and treatment, and imaging-guided chemotherapy [7]. Theranostic platforms often use nanoparticle carriers or prodrugs activated by specific biological stimuli to ensure targeting and to improve patient response times, survival, and quality of life [8]. The combination of precision medicine and theranostics is expected to lead to more effective, patient-specific care by identifying disease-specific targets and delivering therapies that may be monitored continuously for optimal outcomes [9].

Single-domain antibodies (sdAbs; hereafter nanobodies), derived from camelidae, are emerging as powerful tools in targeted therapy due to their unique properties, including small size, high stability, and excellent tissue penetration [10]. These characteristics enable nanobodies to access difficult-to-target sites and deliver therapeutic agents with greater precision compared to conventional antibodies [11]. Their high specificity and ability to be engineered for multiple applications, such as imaging and treatment, make them valuable in developing targeted therapies for cancer, infectious diseases, and autoimmune disorders. Recent studies, for instance, have demonstrated the successful use of nanobodies in delivering targeted drugs and as diagnostic agents in imaging modalities, such as positron emission tomography (PET) scans [12]. Additionally, the rapid production and cost-effectiveness of nanobodies make them attractive for clinical translation [13]. Here, we report a nanobody-based system that allows for fluorescence-based imaging within the near-infrared (NIR) optical window, while providing robust therapeutic efficacy by virtue of doxorubicin release through glutathione (GSH)-mediated disulfide bond cleavage.

As detailed below, the present prodrug design incorporates an azadipyrrromethane boron difluoride (aza-BODIPY) as the imaging moiety. Aza-BODIPY dyes are known for their strong near-infrared fluorescence, excellent photostability, and high quantum yield, making them highly suitable for in vivo imaging applications [14, 15]. These dyes allow for real-time monitoring of drug distribution and activation in the tumor microenvironment, enabling precise therapeutic intervention [16]. Activation of the prodrugs of the present study is mediated by disulfide bond cleavage, which is selectively triggered by the elevated GSH levels typically found in the tumor microenvironment [17, 18]. Upon cleavage, two critical processes occur simultaneously: (1) activation of aza-BODIPY fluorescence, providing a diagnostic signal that confirms prodrug activation, and (2) release of free doxorubicin, a potent cytotoxic agent, directly at the tumor site. This dual functionality, combined with nanobody-based targeting, allows for precise localization and tracking of drug release while minimizing systemic toxicity.

Another key design element of the theranostic prodrug we report here (vide infra) is that it targets $\alpha_v\beta_3$ integrins (hereafter $\alpha v\beta 3$), which are overexpressed in various cancer types and play a pivotal role in tumor angiogenesis and metastasis [19, 20]. These integrins are recognized as promising targets for precision drug delivery due to their limited expression in normal tissues [21]. Conjugating a nanobody-based prodrug to an $\alpha v\beta 3$

ligand provides for selective tumor targeting, enhancing both the therapeutic index and diagnostic accuracy. This targeted approach further reduces off-target effects and improves the efficiency of drug delivery to cancerous tissues [22]. The present study thus serves to highlight the potential benefits of nanobody-based theranostic prodrugs that integrate targeted therapy and real-time imaging.

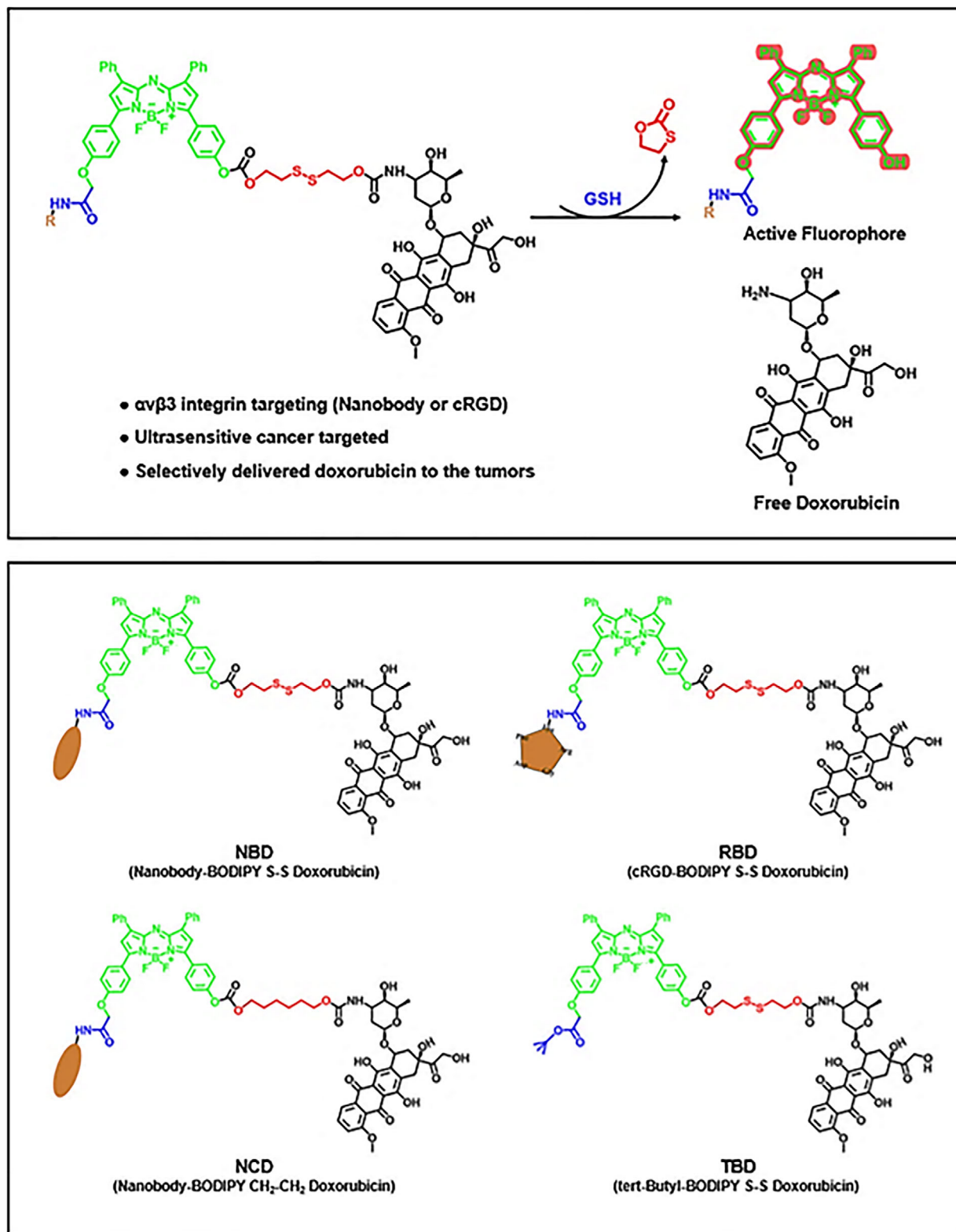
2 | Results and Discussion

2.1 | Design and Synthesis of the Prodrugs

Our objective was to develop a highly efficient and selective theranostic prodrug suitable for in vivo tumor imaging and therapy. With this goal in mind, a BF_2 -chelated azadipyrrromethane-based NIR fluorescent probe was functionalized with a GSH-responsive disulfide (S-S) moiety through a carbamate linker (Scheme 1). This construct was subsequently conjugated to doxorubicin and ultimately linked to a nanobody, yielding the target bioconjugate (**NBD**). Briefly, several key precursors as well as mono-O-tBu-BODIPY were synthesized in accord with reported methods [15]. The tBu-BODIPY-disulfide(S-S)-OH (compound **2**, Scheme 1) was synthesized by reacting mono-O-*tert*-butyl-protected BODIPY (mono-O-tBu-BODIPY) with mono-O-dimethoxytrityl-protected disulfide (mono-O-DMTr-disulfide), followed by removal of the DMTr protecting group. The overall yield of the reaction was 68%. The tBu-BODIPY-disulfide (S-S)-doxorubicin conjugate (**TBD**, Scheme 2) was synthesized by linking the amino group of doxorubicin and compound **2** through a carbamate tether. Deprotection with trifluoroacetic acid then gave compound **3**, which was conjugated to *N*-hydroxysuccinimide in the presence of diisopropylethylamine to form a reactive intermediate (*N*-hydroxysuccinimide ester). This intermediate was subsequently reacted with cRGD to produce **crBD** (Scheme S1). **NCD** was prepared using the same method but starting with compound **4** and mono-O-DMTr-hexanol (Scheme S1). The **TBD**, **NBD**, **crBD** and **NCD** constructs were characterized by ^1H and ^{13}C NMR spectroscopy, as well as by HRMS (Figures S1–S16) and high-performance liquid chromatography (HPLC) analysis. All intermediates were characterized by mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy (Figures S17–S28). Based on Table 1, **NBD** was identified as an optimized design that was expected to combine high-affinity nanobody targeting with a GSH-responsive disulfide linker for efficient intracellular drug release. In contrast, the control conjugates contain only isolated design elements: **NCD** lacks a cleavable linker, **TBD** does not possess a targeting moiety, and **crBD** employs a cyclic peptide with comparatively lower specificity and affinity.

2.2 | Photophysical Properties of Prodrug NBD and its Activation

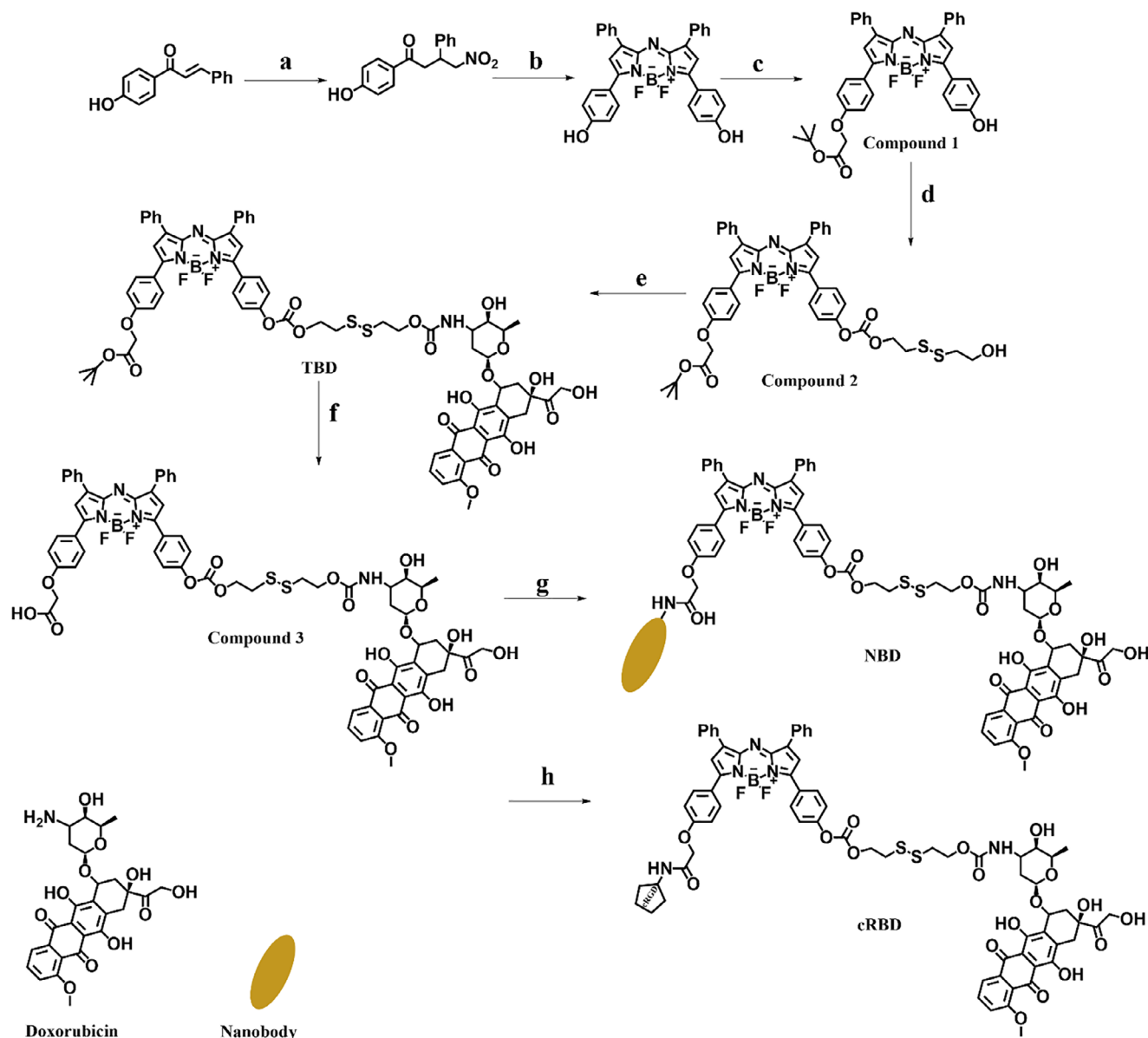
Figure S29 illustrates the DFT-optimized geometries and frontier molecular orbitals of **NBD** (A) and **NCD** (B). In both molecules, a potential intramolecular hydrogen-bonding interaction between the $-\text{BF}_2$ moiety and the adjacent phenyl ring is inferred based on their spatial proximity. The calculated energy gaps between the highest occupied molecular orbital (HOMO) and



SCHEME 1 | Design strategies for nanobody-based theranostic prodrugs (NBD, NCD, cRBD, and TBD). Schematic presentation of drug release from prodrug by glutathione (GSH).

the lowest unoccupied molecular orbital (LUMO) are 2.038 eV for **NBD** and 1.912 eV for **NCD**. The lower HOMO–LUMO gap in **NCD** implies enhanced chemical reactivity and greater sensitivity relative to **NBD**. Computational analysis revealed that **NCD** exhibited a slightly lower HOMO–LUMO gap (1.912 eV)

compared to **NBD** (2.038 eV). This finding is consistent with higher intrinsic electronic reactivity and polarizability as viewed from a theoretical standpoint. However, experimental evaluations under GSH-rich conditions revealed a distinct activation profile, indicating that biological responsiveness is governed not solely



by electronic structure but also by steric and microenvironmental factors. To evaluate the effect of conjugation on nanoparticle size, dynamic light scattering (DLS) analyses were carried out. The hydrodynamic diameter increased from 5.6 nm for the nanobody monomer (**NB**) to 6.7 nm for the **NBD** conjugate, representing an approximately 1.1 nm increase consistent with the expected increase in molecular size due to conjugation of the linker and payload (Figure 1A). Additional DLS measurements of the conjugates (**NBD**, **NCD**, **cRBD**, and **TBD**) performed in PBS containing 5% DMSO are presented in Figure S30A, together with control experiments examining the solvent dependence of the **NBD** hydrodynamic size at different DMSO concentra-

tions (Figure S30B). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis revealed a protein band at around 16 kDa corresponding to the native nanobody (**NB**). The conjugated constructs (**NBD** and **NCD**) displayed comparable band positions without an observable molecular weight shift (Figure 1B). This is attributed to the relatively small molecular mass of the conjugated moieties (~115 Da), which is below the resolution limit of conventional SDS-PAGE for a ~16 kDa protein.

Prodrug **NBD** was expected to be amenable to cleavage as the result of its constituent disulfide (S-S) bonds. Specifically, reaction

TABLE 1 | Rational design and functional characteristics of theranostic conjugates.

Conjugate name	Full form/components	Targeting moiety	Cleavable linker	Functional logic
NBD	Nanobody-disulfide-Doxorubicin	Nanobody (High affinity to $\alpha\beta3$ integrin)	Yes (GSH-sensitive disulfide)	Superior ; Combines high-specificity nanobody targeting with efficient intracellular drug release
NCD	Nanobody-CH ₂ -CH ₂ -Doxorubicin	Nanobody	No (Stable alkyl chain)	Targeting only ; Reaches the cancer cell but cannot efficiently release the free Doxorubicin payload
TBD	Tertiarybutyl-disulfide-Doxorubicin	None (T-butyl group)	Yes (GSH-sensitive disulfide)	Cleavable only ; It can release the drug if it enters a cell but lacks the “homing” ability to find cancer cells specifically
cRBD	cRGD-disulfide-Doxorubicin	cRGD peptide	Yes (GSH-sensitive disulfide)	Standard targeting ; Uses a cyclic peptide for targeting; functional, but usually less potent/specific than the Nanobody (NBD)

with GSH was expected to yield 1,3-oxathiolan-2-one and trigger release of both doxorubicin and the aza-BODIPY fluorophore in their corresponding phenol and hydroxyl forms (Scheme 1). To determine whether GSH would cleave the disulfide moiety in **NBD**, we monitored the changes in the UV-vis absorption spectra and fluorescence emission of the compounds in the presence of various concentrations of GSH under simulated physiological conditions. Initially, we recorded the UV absorbance spectrum of **NBD** dissolved in phosphate-buffered saline (PBS) at different GSH concentrations (Figure 1C). The UV-vis absorption maximum of **NBD** (5 μ M) at 675 nm increased in intensity by approximately 17-fold upon adding 5 mM GSH (Figure S31A). Similarly, the emission intensity at 730 nm increased 35-fold when exposed to 5 mM of GSH (Figure S31B) with the changes proving concentration dependent (Figure 1D). Analysis of **NBD** (5 μ M) by high-performance liquid chromatography (HPLC) in the presence of GSH revealed two new bands, consistent with the GSH-promoted cleavage of the disulfide (S-S) bond to give the phenol form of the aza-BODIPY and free doxorubicin (Dox) (Figure S32).

Additional evidence for the proposed reaction between GSH and **NBD** was obtained through high-resolution mass spectrometry (HR-MS) following incubation with 5 mM GSH at 37°C for 30 min. This analysis revealed a prominent $[M + H]^+$ peak at m/z 15,122.092, corresponding to the activated fluorescent species, and another significant $[M + H]^+$ peak at m/z 544.17, corresponding to doxorubicin (Figure S33).

Prior to testing the **NBD** prodrug in an in vitro cellular system, its selectivity was evaluated against a range of biologically relevant analytes. These included a metal ion (Ca^{2+}), several redox-active species (ascorbic acid, vitamin B6, human serum albumin [HSA], and H_2O_2), representative enzymes (lipase, pepsin, trypsin, esterase, and phosphatase), and selected amino acids (cystine, serine, lysine, histidine, arginine, and tyrosine). **NBD** exhibited no significant spectroscopic response to these interferents, confirming its high selectivity (Figure 1E,F) to GSH. The fluorescence intensity of **NBD** was monitored at an emission

wavelength of 730 nm as a function of GSH concentration (Figure 1G). The fluorescence intensity of **NBD** was monitored as a function of time in the presence and absence of GSH and esterase at an emission wavelength of 730 nm (Figure 1H). The time required to reach fluorescence saturation decreased with increasing concentrations of GSH and esterase, indicating enhanced prodrug activation. In contrast, **NCD** exhibited no significant fluorescence enhancement under identical conditions throughout the incubation period; this finding is consistent with minimal responsiveness to GSH and esterase (Figure 1I). Notably, **NBD** retained its activatable prodrug characteristics, exhibiting only weak fluorescence emission even in polar solvents such as acetonitrile and methanol. This behavior is interpreted in terms of strong intermolecular hydrogen-bonding interactions between individual **NBD** molecules in their activated phenol form, leading to fluorescence quenching. In contrast, dimethyl sulfoxide (DMSO) appeared to stabilize the excited states, thereby enhancing the fluorescence emission (Figure S34A). A similar solvent-dependent fluorescence response was observed for **cRBD** (Figure S34B).

2.3 | Specificity of NBD for $\alpha\beta3$ -Positive Cells

To enhance the selectivity of compound **3** for cancer cells overexpressing $\alpha\beta3$ integrin, we conjugated it to the specific human $\alpha\beta3$ -specific nanobody, a well-established $\alpha\beta3$ -targeting antibody fragment. This conjugation was expected to direct **NBD** specifically to $\alpha\beta3$ -expressing cancer cells while reducing non-specific interactions with healthy cells. To assess the selectivity of **NBD**, we conducted experiments using four different cell lines with varying $\alpha\beta3$ expression levels: SKOV3 (human ovarian cancer cell line; high (++) $\alpha\beta3$ expression), U87MG (human glioblastoma cell line; high (++) $\alpha\beta3$ expression), A431 (human epidermoid carcinoma cell line; minimal (–) $\alpha\beta3$ expression), and A549 (human lung adenocarcinoma cell line; minimal (–) $\alpha\beta3$ expression). Western blot analysis (Figure S35) confirmed these expression patterns, validating the selection of these cell lines for further investigation. The therapeutic efficacy of

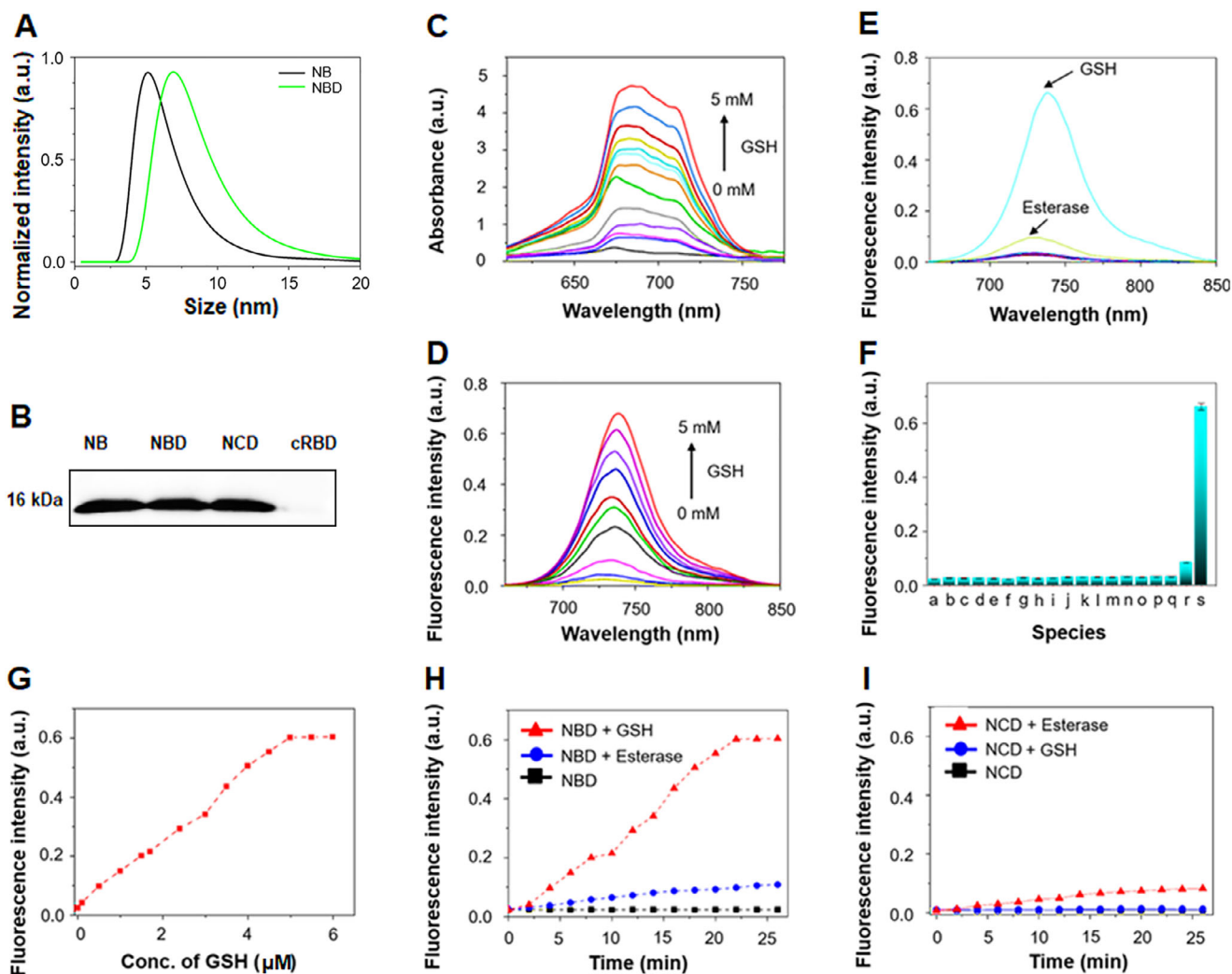


FIGURE 1 | Photophysical characterization and GSH-responsiveness of NBD. (A) Dynamic light scattering (DLS) analysis showing the average hydrodynamic diameters of **NBD**, and **NB** (nanobody monomer) in PBS. (B) SDS-PAGE analysis of the protein confirming the conjugation of nanobodies to prodrug constructs. (C) UV-Vis absorption spectra of **NBD** (5 μ M) in PBS in the presence of increasing concentrations of glutathione (GSH, 0–5 mM). (D) Fluorescence emission spectra of **NBD** ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 685/730$ nm) following 15 min incubation with varying concentrations of GSH at 37°C. (E) Fluorescence response of **NBD** (5 μ M) to a panel of potential serum interferents, including: (a) PBS control, (b) 2.5 mM CaCl_2 , (c) 200 μ M H_2O_2 , (d) 1 mM vitamin C, (e) 1 mM vitamin B6, (f) 100 μ M HSA, (g) 10 mM glucose, (h) 200 U/L lipase, (i) 100 μ g/L pepsin, (j) 200 ng/mL trypsin, (k) 200 U/L phosphatase, (l–q) 1 mM of various amino acids (arginine, serine, histidine, cystine, tyrosine, lysine), (r) 30 U/mL esterase, and (s) 5 mM GSH. (F) Bar graph summarizing the fluorescence intensity of **NBD** in the presence of the same biological species as in (E). Data represent the mean $\pm$ standard deviation (SD) from four independent experiments for the results shown in F. (G) Fluorescence intensity changes of **NBD** (100 nM) after treatment with increasing GSH concentrations (0–6 μ M) for 15 min. (H, I) Time-dependent fluorescence response curves of **NBD** (H) and **NCD** (I) in the presence of GSH or esterase.

disulfide-linked conjugates (**NBD**, **TBD**, and **cRBD**) is dependent on intracellular GSH levels that trigger thiol-disulfide exchange and drug release. Baseline GSH concentrations were quantified in U87MG, SKOV3, A431, and A549 cells (Figure S36). A549 cells exhibited the highest GSH levels (~ 125 nmol/mg protein), indicating a highly reductive intracellular environment. SKOV3 cells showed moderate GSH levels (~ 75 nmol/mg protein), whereas U87MG and A431 cells displayed comparatively lower levels (~ 25 nmol/mg protein). These differences may contribute to variability in GSH-triggered drug release and therapeutic efficacy.

Prior to evaluating the targeting efficiency of **NBD** and **cRBD**, their cytocompatibility was assessed to confirm minimal intrinsic

toxicity in $\alpha\beta 3$ -expressing cell lines. SKOV3, U87MG, A549, and A431 cells were incubated at concentrations ranging from 0 to 25 μ M of **NBD**, **cRBD**, **TBD**, and **NCD** for 1, 3, and 24 h. Cell viability analysis showed that over 60% of cells remained viable following 1 h and 3 h treatments with **NBD** and **cRBD** (Figure S37A–D), as well as with **NCD** and **TBD** (Figure S38A–D). These findings indicate acceptable short-term biocompatibility at lower concentration (0–15 μ M) under physiological conditions. A431 cells, which exhibit minimal $\alpha\beta 3$ integrin expression, showed reduced receptor-mediated internalization of **NBD** compared to $\alpha\beta 3$ -positive SKOV3 and U87MG cells. Although limited time-dependent uptake may occur via non-specific endocytic pathways, this process is less efficient than receptor-mediated

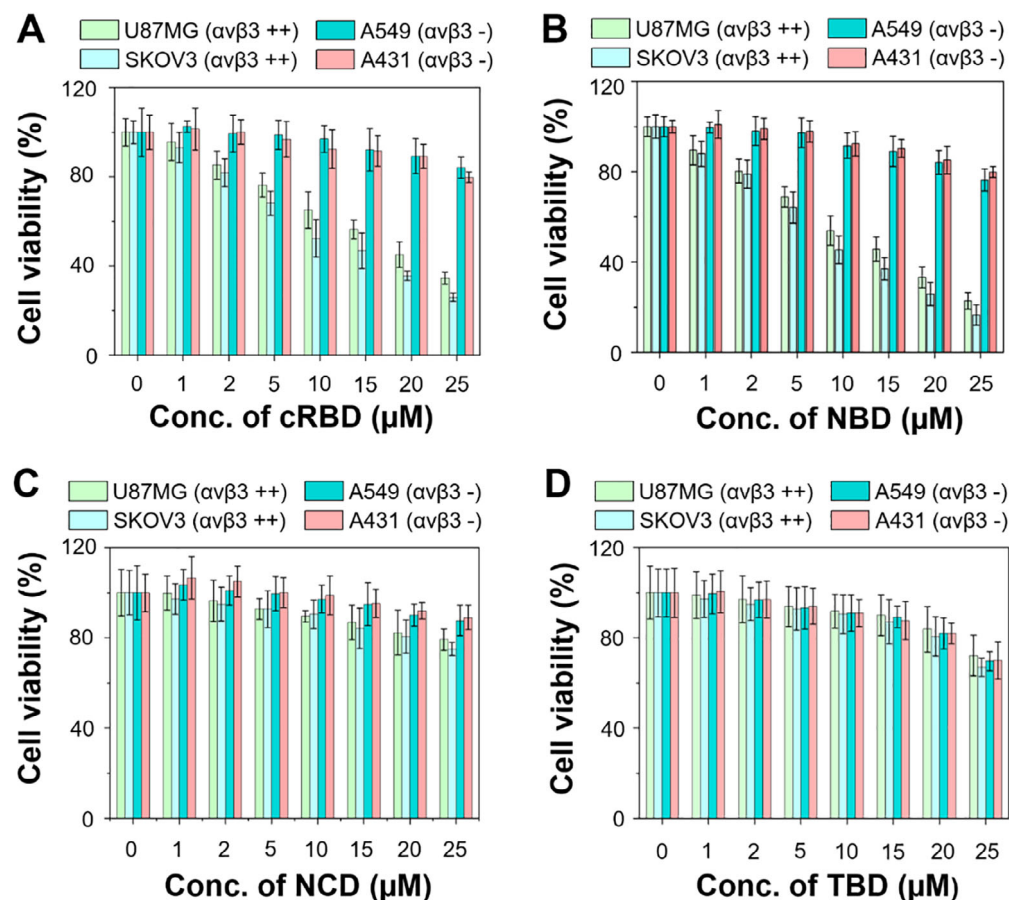


FIGURE 2 | Cytotoxicity evaluation of nanobody-based prodrugs. The antitumor efficacy of nanobody-based prodrugs was evaluated in two $\alpha v \beta 3$ integrin-positive cancer cell lines (SKOV3 and U87MG) and two $\alpha v \beta 3$ integrin-negative cell lines (A549 and A431). Cells were treated with increasing concentrations of prodrugs (A) **cRBD**, (B) **NBD**, (C) **NCD**, and (D) **TBD** for 24 h. Cell viability was determined using the MTT assay. Notably, treatment with **NBD** and **cRBD** resulted in a significant reduction in viability of $\alpha v \beta 3$ integrin-positive cells (SKOV3 and U87MG) compared to $\alpha v \beta 3$ -negative controls (A549 and A431), indicating enhanced cytotoxicity through integrin-mediated selective prodrug delivery.

internalization. Consequently, reduced intracellular accumulation leads to lower cytotoxicity after 24 h incubation.

At 24 h, significant cytotoxicity was observed in $\alpha v \beta 3$ -positive SKOV3 and U87MG cells treated with **NBD** and **cRBD**, whereas $\alpha v \beta 3$ -negative A431 and A549 cells exhibited substantially lower sensitivity (Figure 2A,B). Notably, **NBD** induced greater cytotoxicity than **cRBD** at equivalent concentrations, highlighting the superior targeting capacity of nanobody-based delivery relative to **cRGD**. Given the relatively enhanced reductive capacity of A549 cells and the preferential targeting expected in the case of these cells, it was hypothesized that **NBD** would demonstrate markedly higher cytotoxicity in this line compared to A431. However, quantitative viability assays revealed an unexpected trend. Despite efficient payload release, the cytotoxic impact on A549 cells was attenuated relative to the high drug concentration delivered. This phenomenon is attributed to the differential stress-response pathways activated upon the nuclear translocation of Dox. In A431 cells, the released doxorubicin follows a canonical apoptotic pathway. In contrast, in A549 cells, doxorubicin treatment triggers a robust autophagic response [23, 24]. Thus, **NCD** and **TBD** treatments did not result in marked cytotoxicity even after 24 h in both $\alpha v \beta 3$ -positive and -negative cell lines (Figure 2C,D). These findings lead us to suggest that these prodrugs remain inactive

until disulfide bond cleavage takes place and intracellular release of active doxorubicin occurs. The lack of toxicity in **TBD**-treated cells further indicates limited intracellular delivery in the absence of a targeting moiety.

To evaluate the preferential targeting of $\alpha v \beta 3$ integrin-positive cells by **NBD**, cellular uptake studies were performed using flow cytometry and confocal laser scanning microscopy (CLSM). Flow cytometric analysis of cellular uptake was conducted to quantify cellular uptake (%) following incubation of SKOV3, U87MG ($\alpha v \beta 3$ -positive), and A431 ($\alpha v \beta 3$ -negative) cells with **TBD**, **NBD**, and **cRBD** at concentrations of 5 or 10 μM (Figure 3A–C). **NBD**-treated SKOV3 and U87MG cells exhibited significantly higher cellular uptake compared to **cRBD** and **TBD**, indicating enhanced uptake in $\alpha v \beta 3$ -expressing cells. In contrast, A431 cells showed minimal cellular uptake (%) with **NBD** prodrug consistent with limited nonspecific uptake. Notably, **NBD** demonstrated selectivity comparable to that of **cRBD**, a finding interpreted in terms of both targeting moieties facilitating intracellular drug delivery under in vitro conditions. This is as expected for the putative targeting advantage conferred by the nanobody-conjugated design. Mean fluorescence intensity (MFI) was evaluated by flow cytometry in the $\alpha v \beta 3$ -positive (SKOV3 and U87MG) and $\alpha v \beta 3$ -negative (A431) cell lines following

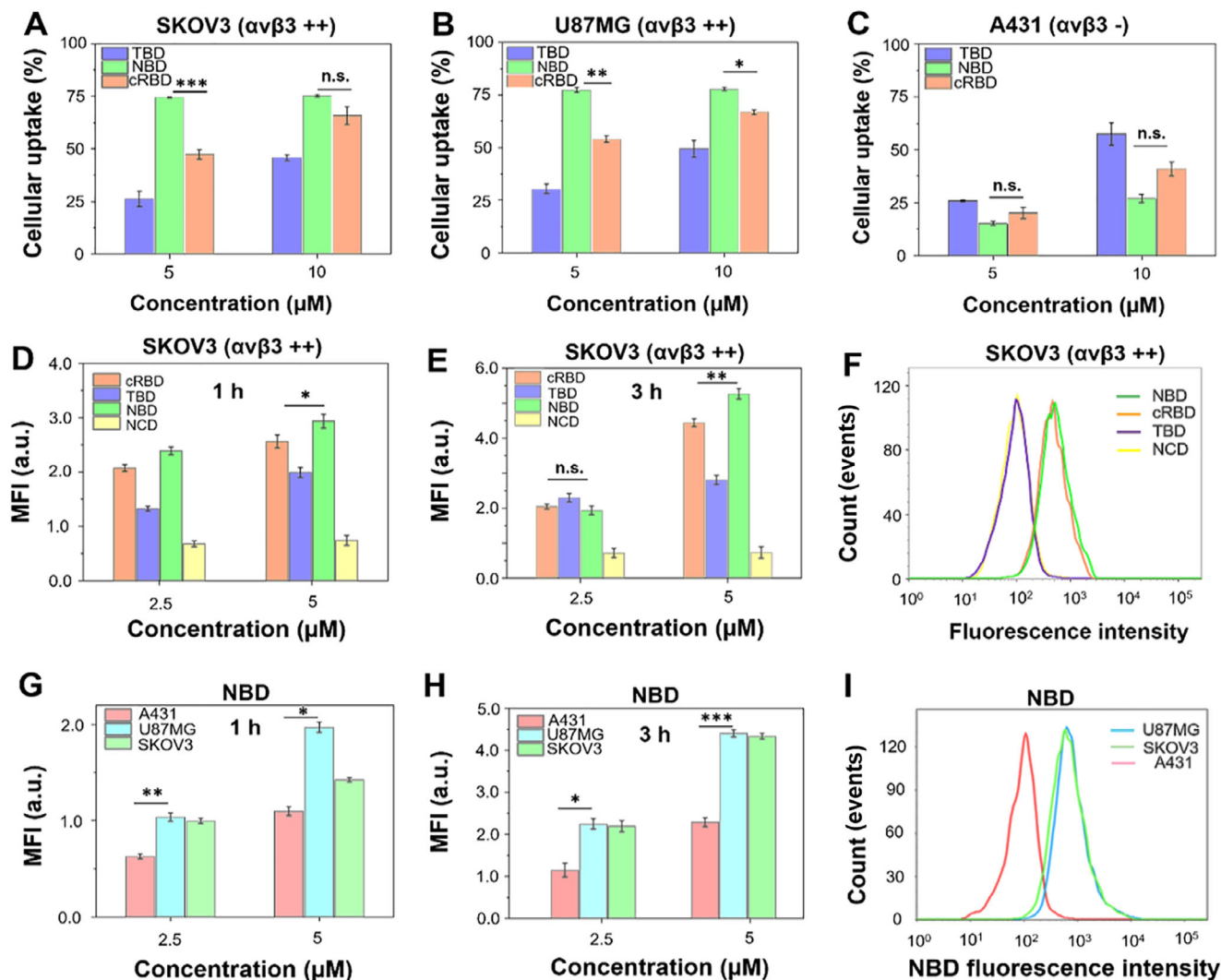


FIGURE 3 | Flow cytometry analysis of cellular uptake of nanobody-based prodrugs. Cellular uptake was assessed by flow cytometry in two $\alpha\text{v}\beta 3$ integrin-positive cancer cell lines, SKOV3 (A) and U87MG (B), as well as the $\alpha\text{v}\beta 3$ integrin-negative cell line A431 (C), following treatment with TBD, NBD, or cRBD (5 or 10 μM). Comparative MFI analyses were further conducted in SKOV3 cells treated with TBD, NBD, cRBD, or NCD (2.5 or 5 μM) for 1 h (D) and 3 h (E). (F) Representative count histograms are presented for SKOV3 cells treated with the same compounds (5 μM) for 3 h. Mean fluorescence intensity (MFI) was quantified in SKOV3, U87MG, and A431 cells treated with TBD, NBD, or cRBD at concentrations of 2.5 or 5 μM for 1 h (G) and 3 h (H). (I) Representative cell count histograms are shown for U87MG, SKOV3, and A431 cells treated with NBD (5 μM) for 3 h. Data represent the mean $\pm$ SD from three independent experiments. Statistical analysis was performed using an unpaired Student's t-test; $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***)

treatment with cRBD, TBD, NBD, and NCD at concentrations of 2.5 or 5 μM for 1 h and 3 h. SKOV3 and U87MG cells, which exhibit high $\alpha\text{v}\beta 3$ integrin expression, showed markedly enhanced fluorescence signals in a time-dependent manner, indicating more efficient internalization of NBD compared to its controls (Figure 3D,E). The cell count histograms further supported this observation, with a similar trend observed in the count graph (Figure 3F). Cellular uptake analysis revealed a cell-type-dependent variation in internalization efficiency. At both tested concentrations (5 and 10 μM), uptake followed a consistent hierarchical trend: U87MG $\sim$ SKOV3 $>$ A549 $>$ A431 (Figure S39). While NBD exhibited enhanced uptake in high $\alpha\text{v}\beta 3$ -expressing cell lines, the magnitude of improvement relative to cRBD was moderate. Nevertheless, both constructs demonstrated preferential internalization in receptor-enriched models compared to low-expressing cells, supporting receptor-

mediated uptake. Overall, NBD showed selectivity comparable to that of cRBD under in vitro conditions, indicating that the nanobody-conjugated design effectively mediates targeted intracellular delivery. Furthermore, we measured the MFI of A431, U87MG, and SKOV3 cells treated with NBD at concentrations of 2.5 or 5 μM for 1 and 3 h (Figure 3G,H). The results demonstrated a time-dependent increase in fluorescence intensity that positively correlated with $\alpha\text{v}\beta 3$ integrin expression levels, with SKOV3 and U87MG ($\alpha\text{v}\beta 3$ -positive) exhibiting significantly higher signals compared to A431 ($\alpha\text{v}\beta 3$ -negative). The cell count histograms further corroborated this trend, showing a consistent shift in cell populations corresponding to increased fluorescence, as illustrated in the count graph (Figure 3I). A431 cells ($\alpha\text{v}\beta 3$ -negative) exhibited negligible fluorescence, a finding consistent with minimal uptake of the nanobody conjugate prodrug NBD.

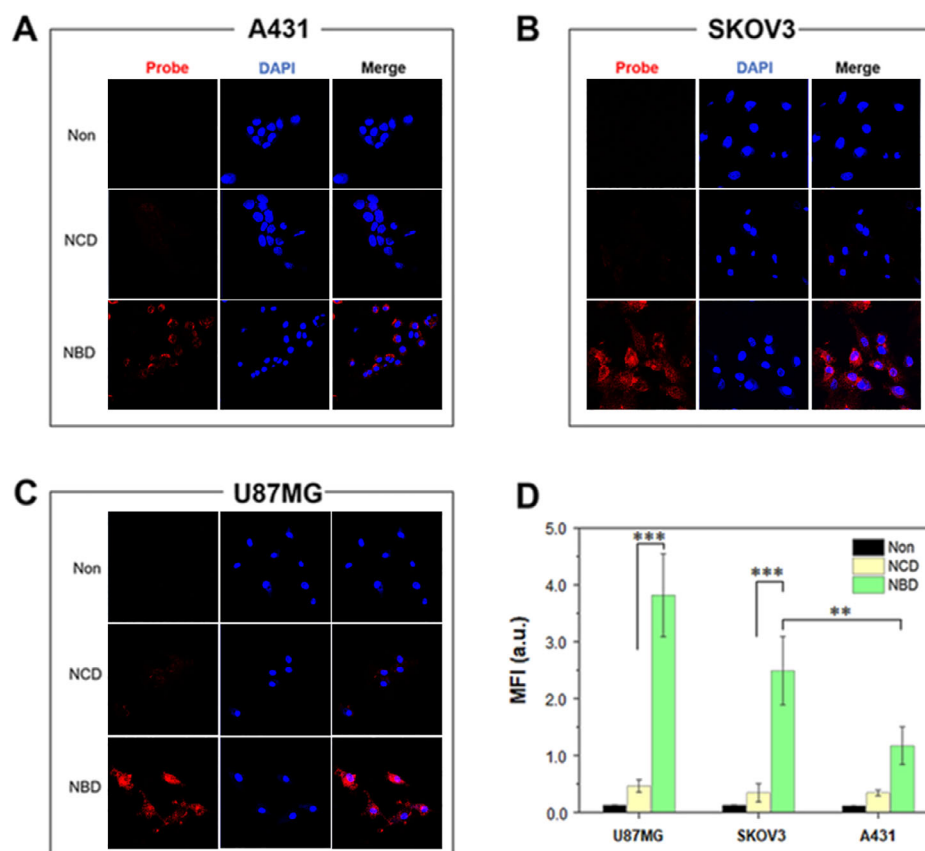


FIGURE 4 | In vitro CLSM imaging of nanobody-based prodrugs in cancer cells. Confocal laser scanning microscopy (CLSM) was used to assess cellular uptake in one $\alpha\beta$ integrin-negative cell line of A431 (A) and two $\alpha\beta$ integrin-positive cancer cell lines of SKOV3 (B) and U87MG (C), following treatment with **NBD** or **NCD** (5 μ M) for 15 min. Imaging was performed using the Alexa 647 filter set ($E_x/E_m = 650/671$ nm). (D) Quantitative analysis of intracellular fluorescence intensity was conducted using the ImageJ software on 15 individual cells per group. Statistical significance was determined using an unpaired Student's t-test; **, $p < 0.01$; ***, $p < 0.001$.

These findings were further validated by CLSM, which revealed that $\alpha\beta$ -positive U87MG and SKOV3 cells exhibited markedly higher fluorescence intensities following incubation with 5 μ M **NBD** compared to **NCD**. In contrast, A431 cells, which lack $\alpha\beta$ expression, displayed significantly lower fluorescence (Figure 4A–D). These results confirm the high selectivity of **NBD** for $\alpha\beta$ -overexpressing cells. The MFI was enhanced approximately 7.5-fold in $\alpha\beta$ -overexpressing cells when the cells were incubated with **NBD** compared to **NCD** (Figure 4D). The observed increase in MFI is attributed to the GSH-mediated cleavage of the disulfide bonds, leading to prodrug release and activation.

To investigate further the impact of carrier specificity and size on intracellular delivery, we compared nanobody-BODIPY-S-S-Dox (**NBD**) and antibody-BODIPY-S-S-Dox (**ABD**) (Figure S40). In SKOV3 cells, **NBD** induced a concentration-dependent decrease in cell viability, reaching approximately 58% viability at 25 mM (Figure S40C). In contrast, despite measurable cellular uptake, **ABD** exhibited reduced cytotoxicity, retaining nearly 80% viability at the same concentration. In A431 cells, both constructs showed minimal cytotoxicity, with viability remaining above 80% across the tested concentration range (Figure S40D).

In addition to viability assessment, flow cytometric analyses were performed to quantify cellular internalization. In SKOV3

cells, the **NBD** construct demonstrated markedly superior internalization, achieving approximately 75% uptake at both 5 μ M and 10 μ M, which was substantially higher than that observed for **ABD** (~27% and ~47%, respectively; Figure S40E). Conversely, in A431 cells, **ABD** exhibited slightly higher uptake (~25% and ~46%) compared to **NBD** (~19% and ~30%; Figure S40F). The observed disparity in uptake between the **NBD** (~16 kD) and **ABD** (~150 kD) constructs underscores the influence of carrier size and targeting specificity on cellular internalization efficiency. The significantly enhanced uptake of the smaller **NBD** construct in SKOV3 cells leads us to suggest that nanobody-based targeting may benefit from reduced steric hindrance and improved accessibility to $\alpha\beta$ epitopes, thereby enabling more efficient receptor engagement and endocytosis compared to the considerably larger IgG-based system. Interestingly, this trend was modestly reversed in A431 cells, where **ABD** exhibited slightly greater uptake. This difference may reflect the low $\alpha\beta$ expression profile of A431 cells, in which non-specific interactions or Fc-mediated processes may contribute more prominently than receptor-specific internalization. Collectively, these results reinforce the importance of epitope-specific targeting in achieving efficient intracellular delivery and GSH-responsive drug release, and they further support the mechanistic advantage of nanobody-based constructs over non-specific antibody conjugates in $\alpha\beta$ -positive cancer models.

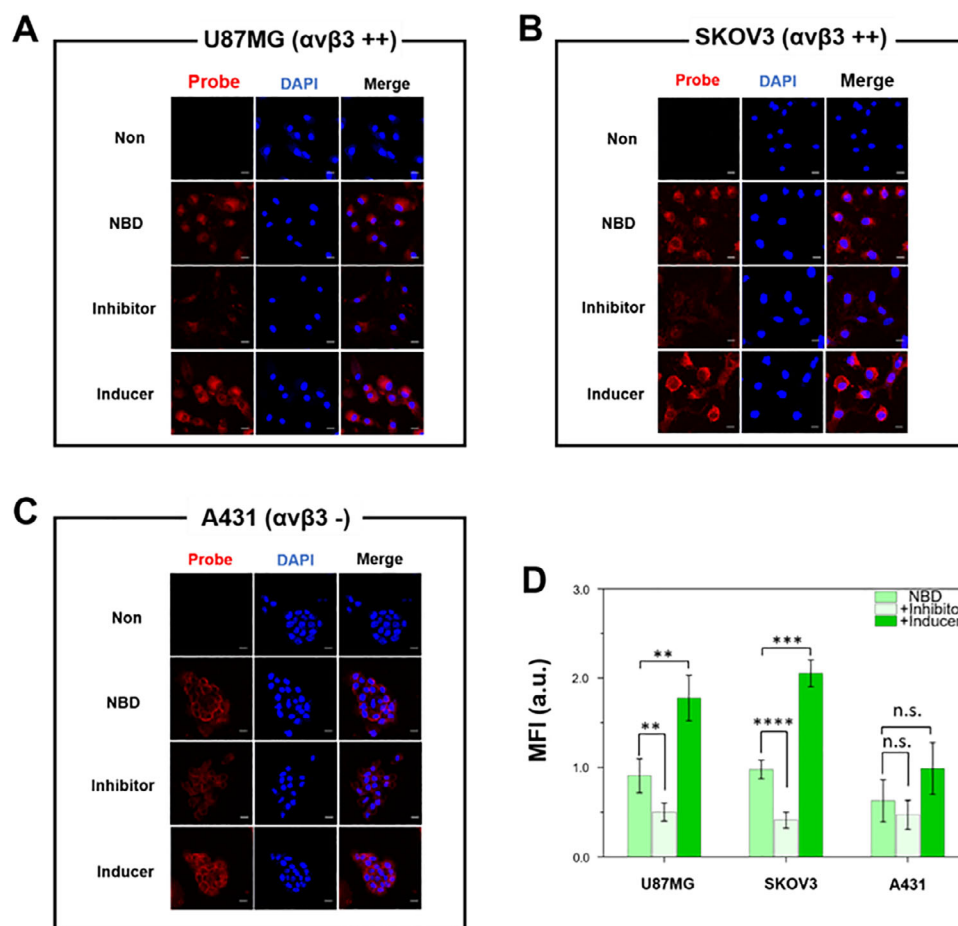


FIGURE 5 | NBD imaging of endogenous GSH levels in live cells under redox-modulating conditions. (A–C) Confocal laser scanning microscopy (CLSM) and corresponding brightfield overlay images of U87MG (A), SKOV3 (B), and A431 (C) cells stained with **NBD** (5 μ M), followed by treatment with either the GSH inducer N-acetylcysteine (5 mM, 1 h) or the GSH inhibitor maleimide (20 μ M, 30 min) at 37°C under humidified conditions. After treatment, cells were washed with PBS and imaged. (D) Quantitative analysis of intracellular fluorescence intensity was performed using the ImageJ software. Data represent the mean $\pm$ SEM from three independent samples. Statistical analysis was performed using an unpaired Student's t-test; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

To evaluate the capability of **NBD** to detect endogenous GSH levels, we conducted experiments in U87MG, SKOV3, and A431 cells, following treatment with N-acetylcysteine (NAC), a known GSH inducer. Cells were first stained with **NBD** (5 μ M) for 15 min, followed by incubation with NAC (5 mM, 1 h) or the GSH inhibitor maleimide (20 μ M, 30 min) at 37°C. After washing, cells were imaged using CLSM (Figure 5). CLSM analysis revealed a significant increase in intracellular fluorescence intensity upon NAC stimulation. Specifically, U87MG cells showed a $\sim$ 2-fold increase compared to untreated controls, whereas maleimide treatment led to a $\sim$ 1.8-fold reduction in fluorescence intensity (Figure 5A). A similar pattern was observed in SKOV3 cells, where NAC treatment resulted in a $\sim$ 2.4-fold fluorescence increase, and maleimide reduced the signal by $\sim$ 2.2-fold compared to non-treated cells (Figure 5B). In contrast, A431 cells exhibited 30–40% of the maximum fluorescence intensity observed in $\alpha\beta$ 3-positive cell lines, consistent with their low integrin expression. Treatment with NAC or maleimide induced modest but measurable changes in the fluorescence, leading us to suggest partial involvement of intracellular redox processes independent of strong receptor-mediated uptake (Figure 5C). These findings are taken as evidence that **NBD** is capable of detecting

changes in endogenous GSH levels within $\alpha\beta$ 3-positive cells. The enhancement in fluorescence following NAC treatment further supports the probe's activation through GSH-mediated disulfide bond cleavage. Quantitative CLSM analysis confirmed that fluorescence intensity increased substantially with NAC-induced GSH upregulation, while control groups displayed relatively weak fluorescence (Figure 5D).

2.4 | Targeting and Precise Long-Time Retention of **NBD** In Vivo

To assess the potential of **NBD** as a tumor-targeting theranostic prodrug for cancer treatment, we conducted experiments using a SKOV3 tumor xenograft mouse model. After establishing the tumor model, the prodrugs of this study (**TBD**, **NBD**, **cRBD**) and Dox (5 mg/kg) were administered intravenously (IV) to the mice through their tail veins. The long-term retention of nanobody-attached **NBD** in vivo was observed, with **BODIPY**-derived fluorescence signals seen at the tumor site for over 72 h. While extended retention of **NBD** can enhance therapeutic efficacy, it also raises concerns regarding clearance difficulties,

potential phototoxicity to organs, and side effects from excessive drug accumulation. However, the rapid clearance of **NBD** from non-target organs within 24 h in the absence of illumination (Figure 6A) lends credence to the notion that conjugation of nanobody with $\alpha v \beta 3$ integrin improved clearance efficiency compared to **crBD**. This improvement is attributed to the high specificity and affinity of the nanobody ($K_D = \sim 9.4$ nM; US Patent No. 12,383,634, 2025) [25], used in this study, toward tumor-associated integrins, facilitating targeted accumulation and subsequent elimination. In contrast, **TBD** and Dox, lacking such a targeting moiety, may exhibit prolonged circulation and nonspecific distribution, potentially leading to reduced therapeutic precision and increased off-target effects. These results underscore the importance of rational ligand design in optimizing the balance between tumor retention and systemic clearance for nanobody-based prodrugs. This observation highlights the critical role that active targeting can play in modulating the in vivo pharmacokinetics of theranostic agents.

After injection of these prodrugs, we observed fluorescent signal enhancement primarily at the tumor sites by **NBD** (indicated by the white circle) around 1.9-fold higher than **crBD**, and around 6.8-fold than **TBD** after 1 day. After 2 days, it was around 2- and 4.9-fold higher than **crBD** and **TBD**, respectively. After 3 days, the fluorescence signal intensity at the tumor sites produced by **NBD** was around 1.7-fold higher than **crBD** (Figure 6B). This observed enhancement is thought to reflect the selective activation and retention of the **NBD** prodrug at the tumor sites.

To investigate further the above suggestion, the biodistribution of prodrugs in this study (**TBD**, **NBD**, **crBD**) and Dox were evaluated in mice bearing SKOV3 tumors 3 days after injection (Figure 6C). Phosphate buffered saline (PBS) was used as a control. After sacrifice, selected organs and tumor tissues were sampled from the mice and used to compare their respective fluorescence intensities. Solid tumors from the animals in **PBS**, **TBD** and Dox groups ($n = 5$ each) showed no noticeable fluorescence, whereas obvious fluorescence was seen in the tumors of mice treated with **NBD**. The strong signal observed in the mouse stomach and liver in these ex vivo experiments is attributed to the nonspecific accumulation of **crBD**. We detected relatively weaker fluorescence signals at the tumor tissue in the **crBD** group. Quantitative analysis revealed a 4.1-fold higher fluorescence intensity for **NBD** compared to **crBD** in the tumor region (Figure 6D). To validate further the tumor-targeting specificity of **NBD** in comparison to **crBD**, **TBD**, and free Dox, we employed a SKOV3 xenograft tumor model. Mice bearing subcutaneous tumors were intravenously injected with equimolar concentration (0.25 mM) of each prodrug. After 24 h, strong fluorescence signals were observed at the tumor site (indicated by white circles) in the **NBD**-treated group compared to those treated with **crBD** or **TBD** (Figure S41A), indicating enhanced tumor accumulation.

Ex vivo imaging of excised tumors from five treatment groups (**PBS**, Dox, **TBD**, **NBD**, and **crBD**) further corroborated these findings. Tumors from **NBD**-treated mice displayed a ~ 3 -fold increase in fluorescence intensity compared to those treated with **crBD** (Figure S41B). Quantitative analysis revealed that fluorescence intensity in the **NBD** group was approximately 2.9-fold higher than **crBD** group, and nearly 7-fold higher than

TBD group within the tumor region (Figure S41C). These results collectively confirm the superior tumor-targeting ability and selective accumulation of **NBD**, likely attributable to nanobody-mediated recognition of $\alpha v \beta 3$ integrin overexpressed in SKOV3 tumors. Dissection analysis at 24 h confirmed concentrated fluorescence within the tumor tissue with minimal signals in other organs, indicating that **NBD** was precisely activated and exhibited sustained retention at the tumor site upon activation (Figure S42). To assess the in vivo distribution and tumor accumulation of the prodrugs, tumor-bearing mice were intravenously administered with **PBS**, **TBD**, **NBD**, **crBD**, or free Dox (0.5 mM, 100 μ L). Confocal microscopy images of H&E-stained tumor sections were acquired from the five treatment groups (Figure S43). Compared to the other formulations, the **NBD**-treated group exhibited markedly stronger fluorescence signals in both the Dox and BODIPY channels, indicating enhanced tumor accumulation and prolonged retention. Notably, the merged images revealed intense co-localization Dox and BODIPY signals within the same tumor regions, suggesting efficient delivery and intracellular activation of the nanobody-conjugated prodrug. Furthermore, the biodistribution of doxorubicin was evaluated using HPLC. Mice were sacrificed at 24 h after treatments. Several organs and tumor tissues of equal weight were harvested and processed into homogenates for HPLC analysis. As shown in Figure 6E and Figure S44, the concentration of doxorubicin in the tumor tissues reached approximately 4.7 μ M in the **NBD**-treated group, compared to 2.2, 1.5, and 0.8 μ M in the **crBD**-, **TBD**-, and free Dox-treated groups, respectively. These results demonstrate that the nanobody-conjugated prodrug **NBD** enables markedly enhanced tumor accumulation of the payload doxorubicin, thereby supporting its superior tumor-targeted delivery and potential for effective cytotoxic activity.

2.5 | Anti-Cancer Effect of NBD In Vivo

Building upon the above findings, the tumor-specific in vitro cellular uptake, imaging performance, antiproliferative activity, and biodistribution of the prodrugs (**TBD**, **NBD**, **crBD**, and Dox) were evaluated. Encouraged by the strong tumor-targeting capability and prolonged intratumoral retention of **NBD**, its therapeutic efficacy was further studied in comparison to that of **TBD**, **crBD**, and free Dox in tumor-bearing mice (treatment started at an initial tumor volume of ~ 120 mm³), as illustrated in Figure 7A. In this study, mice ($n = 10$ in each group) were administered with the prodrugs (5 mg/kg, 100 μ L per dose) via tail vein injection on days 12, 14, 16, and 19 post-tumor inoculation. As shown in Figure 7C, **NBD** treatment led to a significant reduction in tumor volume—approximately 2.1-fold lower compared to the **PBS** and ~ 1.8 -fold decrease compared to other treatment groups by day 46 ($p < 0.001$). Corresponding tumor weights (Figure 7D) confirmed the superior tumor growth inhibition achieved by **NBD**. To assess systemic safety, body weights of the mice were monitored throughout the treatment period, in which no significant weight loss or abnormal physiological changes were observed across the groups, indicating good in vivo tolerability of all tested formulations within the doses used in this study (Figure 7B). These findings reinforce the biocompatibility and in vivo applicability of **NBD**. Additionally, the therapeutic performance of **NBD** was evaluated in a separate cohort of mice bearing SKOV3 cells with smaller initial tumor volumes (~ 85

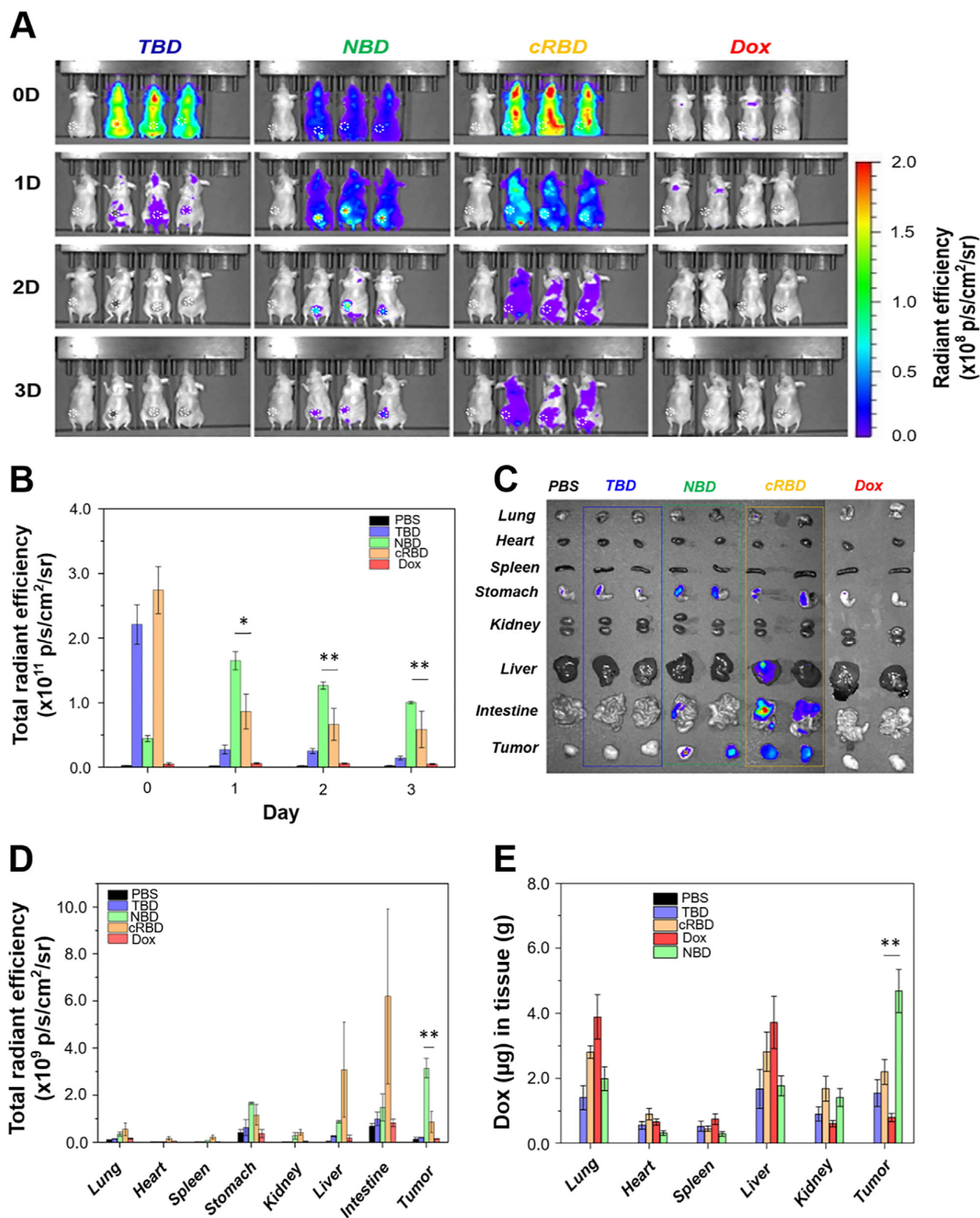


FIGURE 6 | Integrin-targeted prodrugs exhibit sustained retention in tumor sites as visualized by NIR fluorescence in a mouse xenograft model. (A) Real-time tracking of TBD, NBD, cRBD, and Dox within several days. (B) Fluorescence intensity of the tumor site over time after the intravenous injection of TBD, NBD, cRBD, and Dox (5 mg/kg, 100 μ L). (C) Representative ex vivo fluorescence images obtained from several organs, including the lung, heart, spleen, stomach, kidney, liver, intestine, and tumor, extracted after the final in vivo imaging after 3 days. for the NBD group compared to the cRBD, TBD, Dox and PBS group. (D) Relative distribution of fluorescence intensities in tumor and organ sites. (E) Biodistribution profile of TBD, NBD, cRBD, and Dox (0.25 mM, 100 μ L) in mice ($n = 5$) at 24 h post-intravenous injection, highlighting drug accumulation in tumor and major organs by HPLC. Error bars show standard deviations (nonpaired Student's t -test). *, $p < 0.05$; **, $p < 0.01$.

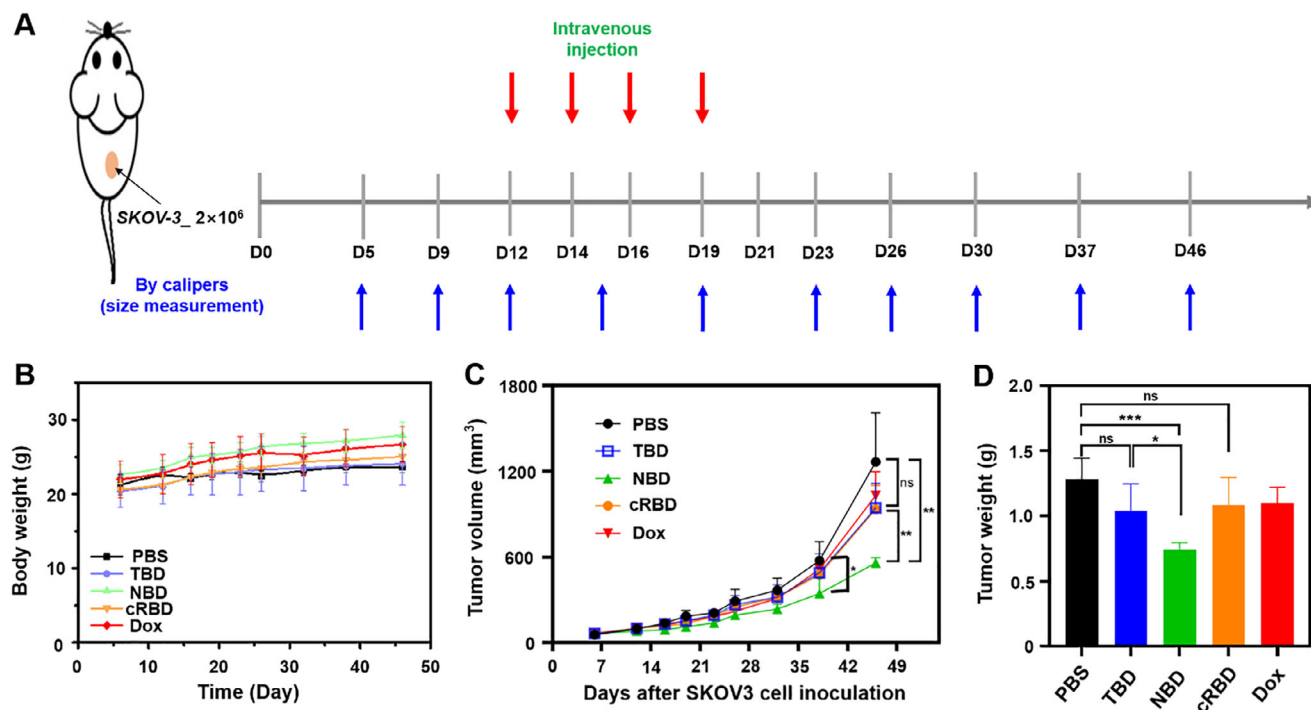


FIGURE 7 | Therapeutic efficacy of NBD, cRBD, TBD, and Dox in a SKOV3 xenograft mouse model. (A) Experimental scheme tumor model preparation and therapeutic schedule for $\alpha v \beta 3$ integrin-targeted prodrugs. (B) Body weight changes in mice over 44 days in different groups ($n = 10$ mice per group). (C) Tumor volume progression in SKOV3 tumor-bearing mice following treatment with various prodrugs (5 mg/kg, 100 μ L; $n = 10$ per group). (D) Tumor weights measured on day 44 post-treatment across different groups ($n = 10$ mice per group). By the study endpoint (44 days), the final tumor weight in mice treated **NBD** was significantly lower than that in mice treated with **TBD**, **cRBD**, and **Dox**. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

mm³), as shown in Figure S45A. The same dosing schedule was applied, with treatments initiated on day 10 post-inoculation. As shown in Figure S45B, **NBD**-treated mice exhibited a ~2.1-fold reduction in tumor volume compared to other groups by day 40 ($p < 0.001$). Collectively, these results demonstrate that **NBD** enables effective tumor suppression in both early- and late-stage treatment models, beneficial effects ascribed to its precise tumor-targeting capacity, enhanced cellular uptake, and sustained therapeutic action.

A potential limitation of directly conjugating a linker–payload to a nanobody for intracellular delivery is related to its inherently small molecular size. While nanobody–drug conjugates may exhibit efficient tumor penetration and rapid cellular internalization in vitro—sometimes even showing advantages over full-length antibody-based constructs—their short blood half-life in vivo can significantly limit systemic exposure and therapeutic efficacy. Rapid renal clearance due to the low molecular weight (~15–16 kDa) reduces circulation time and may compromise target accumulation under physiological conditions. To address this pharmacokinetic constraint, several strategies have been explored, including the use of engineered nanobody constructs with serum albumin-binding domains to enable transient association with circulating albumin, thereby extending plasma half-life [26]. Alternatively, increasing the molecular size through fusion to an antibody Fc domain or incorporation into Fc-containing antibody formats has been investigated to improve FcRn-mediated recycling and prolong systemic retention [27, 28]. Further studies incorporating these half-life extension strategies are warranted to optimize in vivo stability, biodistribution,

and therapeutic performance of nanobody-based linker–payload conjugates.

While cyclic RGD peptides are widely used for integrin targeting, their binding promiscuity across multiple integrin subtypes can result in off-target interactions. In contrast, antibodies and nanobodies provide exceptional epitope specificity [29], enabling selective recognition of $\alpha v \beta 3$ integrin. Furthermore, nanobodies possess unique structural features that allow access to recessed or conformational epitopes inaccessible to small peptides, resulting in higher binding affinity and specificity [30]. Combined with superior stability and resistance to proteolytic degradation, these properties contribute to improved tumor-to-background contrast and enhanced targeting precision in vivo. The nanobody-based theranostic prodrugs developed in this study address critical challenges in cancer therapy, including off-target toxicity and real-time monitoring of therapeutic outcomes. The integration of diagnostic imaging ensures precise treatment delivery and efficacy evaluation. Our results highlight the potential for clinical translation, although further studies are needed to optimize pharmacokinetics and validate safety profiles.

3 | Conclusion

This study presents the successful design and development of a nanobody-based theranostic prodrug that exhibits both robust anticancer efficacy and integrated imaging functionality. By leveraging the high specificity of nanobodies for tumor-associated $\alpha v \beta 3$, the prodrug system enables selective accumula-

tion at the tumor site, thereby enhancing therapeutic precision and minimizing off-target effects. Simultaneously, the built-in diagnostic capability allows for real-time tracking of drug distribution and tumor response, providing valuable insights into treatment dynamics. The modularity of this platform permits adaptation to other tumor markers, potentially broadening its applicability across various cancer types. Collectively, these findings underscore the potential of nanobody-guided theranostic systems to bridge the gap between targeted therapy and non-invasive monitoring. This integrated approach marks a significant advance toward personalized and image-guided cancer treatment strategies in the field of precision oncology.

4 | Experimental Section

4.1 | Materials and Instrumentation

Analytical-grade chemicals were obtained from commercial suppliers and used without further purification. Detailed synthetic procedures are provided in the Supplementary Information. The following reagents were used as received: 4-hydroxychalcone (TCI), N,N-diethylamine (Sigma-Aldrich), nitromethane (TCI), $\text{BF}_3 \cdot \text{OEt}_2$ (Sigma-Aldrich), ammonium acetate (Sigma-Aldrich), 2-hydroxyethyl disulfide (Sigma-Aldrich), 4-nitrophenyl chloroformate (Sigma-Aldrich), 4,4'-dimethoxytrityl chloride (Sigma-Aldrich), N,N-diisopropylethylamine (DIPEA; Sigma-Aldrich), triethylamine (TEA; Sigma-Aldrich), doxorubicin hydrochloride (Dox, Carbosynth), cyclo(-RGDfK) trifluoroacetate (BLD pharma), nanobody against specific human integrin $\alpha v \beta 3$ (Creative Biolabs), human IgG1 monoclonal antibody (Bio-Rad), dimethylformamide (DMF; Sigma-Aldrich), ethanol (Merck), dichloromethane (DCM; Sigma-Aldrich), dimethyl sulfoxide (DMSO; Sigma-Aldrich), and plasma (Sigma). Column chromatography was conducted using silica gel 60 (70–230 mesh), while analytical thin-layer chromatography (TLC) employed pre-coated silica gel 60 plates (0.25 mm thickness). Compound characterization was performed using ^1H and ^{13}C NMR spectroscopy and high-resolution electrospray ionization mass spectrometry (HR-MS/ESI). NMR spectra were recorded on a 400 MHz Bruker spectrometer, using tetramethylsilane (TMS, 0 ppm) as the internal standard and $\text{DMSO}-d_6$ or CDCl_3 as the solvent.

4.2 | Ethics Statement

Male BALB/c nude mice (6 weeks old) were purchased from Narabiotech (Seoul, Korea) and weighed approximately 20–22 g at the time of experimentation. All animal experimental procedures were conducted in accordance with institutional guidelines and were approved by the Institutional Animal Care and Use Committee of the Korea Basic Science Institute (KBSI-IACUC-24-32). The study adhered to established ethical standards for animal research and testing. The maximum tumor volume allowed in our experiments was maintained below 1800 mm^3 , staying within the committee's approved limit of 2000 mm^3 . Mice were housed in a specific pathogen-free (SPF) environment at the Laboratory Animal Center of Korea Basic Science Institute under controlled conditions: temperature of 22°C – 26°C , relative humidity of 50%–60%, and a 12-hour light/dark cycle.

4.3 | Mass Spectrometry Experiments

Electrospray ionization high-resolution mass spectrometry (ESI-HRMS) analysis for organic molecules was conducted using a Synapt G2-Si HDMS system (Waters, USA). The molecules were prepared in methanol at a concentration of $5 \mu\text{M}$ before analysis. Data acquisition and processing were performed using MassLynx software (Version 4.1, Waters, USA), and the results are presented in the Figures of the [Supporting Information](#). For the nanobody-conjugated prodrugs (**NBD** and **NCD**), HPLC coupled with high-resolution mass spectrometry (HPLC–HRMS) was performed using an LTQ Orbitrap XL system (Thermo Scientific, USA). Samples (2 mg/mL , $n = 1$) were dissolved in MES buffer before analysis. Liquid chromatography (LC) separation was carried out on a Hypersil Gold C18 column, using a gradient of acetonitrile and water (0.1% formic acid) ranging from 5% to 70%. The LC flow rate was set at 0.3 mL/min , with a total runtime of 30 min. Protein mass spectrometry data were processed and deconvoluted using the complete protein analysis module in BioPharma Finder 2.0 software (Thermo Fisher, USA).

4.4 | Computational Details

All calculations were performed using the DFT [31] as implemented in the Gaussian 09 suite of programs [32]. All computations were performed on domestic servers at KAIST. Geometry optimizations were performed using the B3LYP functional [33, 34] with Grimme's D3 [35] correction, and 6–31G** for all other atoms [36].

4.5 | Cell Lines

The A431 (human skin cancer) cell line was a kind gift from Dr. Tae Don Kim (Korea Research Institute Bioscience and Biotechnology, South Korea). SKOV3 (ovarian carcinoma, catalog number HTB-77), A549 (lung adenocarcinoma, catalog number CCL-185), and U87MG (human glioblastoma, catalog number HTB-14) cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). To prepare the complete growth medium, the following components were added to the base medium before use: DMEM (A431 and U87MG) and McCoy's (SKOV3) media supplemented with 10% (v/v) FBS and 1% antibiotic (v/v) in a humidified 5% CO_2 incubator at 37°C .

4.6 | Western Blot Analysis

Cells (5×10^6) were harvested and washed three times with PBS before resuspension in lysis buffer (50 mM Tris, 150 mM NaCl, 0.2% Triton X-100, and $10 \mu\text{g/mL}$ PMSF; pH 8.0). After lysis on ice for 20 min, the protein concentrations were quantified using the BCA assay. Equal amounts of proteins ($20 \mu\text{g}$) were separated on a 10% SDS-PAGE gel and transferred onto PVDF membranes. Membranes were then blocked with 5% skim milk for 1 h and incubated overnight at 4°C with mouse anti- $\alpha v \beta 3$ integrin monoclonal antibodies (Proteintech; 1:20,000 dilution in 2% skim milk). After washing with TBST three times, the membranes were incubated with a horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Proteintech; 1:20 000 dilution) for 1 h at room temperature. The membranes were washed three times with TBST buffer, then protein bands were visualized using an ECL detection kit and imaged with a ChemiDoc XR+ system (Bio-Rad).

4.7 | Cell Imaging

Cells were seeded at 2×10^4 per well in 8-well chamber slides and incubated at 37°C for 24 h. The next day, cells were treated with $5 \mu\text{M}$ prodrugs (**NBD** or **NCD** or **TBD**) for 15 min, followed by a 30-minute incubation with either $20 \mu\text{M}$ of the glutathione (GSH, inducer) or maleimide (inhibitor) at 37°C . After washing with DPBS, cells were stained with 4',6-diamidino-2-phenylindole (DAPI) for nuclei visualization and imaged using a CLSM (LSM710; Carl Zeiss, Oberkochen, Germany) equipped with an Alexa 647 filter ($E_x/E_m = 650/671 \text{ nm}$).

4.8 | Flow Cytometry

Cells (1×10^5) were seeded into 6-well plates at 1.5×10^5 cells/well and allowed to be attached overnight. The following day, the cells were treated with 1 μ M prodrugs for 1 or 3 h, washed with PBS, detached, and resuspended in PBS. Red fluorescence was measured using a CytoFLEX flow cytometer (Beckman Coulter) with excitation at 638 nm and emission at 740/80 nm (RL3 channel). A minimum of 10 000 cells per sample were analyzed in triplicate.

4.9 | Cell Viability Assays In Vitro

Cell viability was assessed using the MTT assay. Cells were seeded at 1×10^5 per well in 96-well plates and cultured in DMEM containing 10% FBS for 24 h. Cells were then treated with test compounds (0–25 μ M) and incubated for 1, 3, or 24 h. After drug exposure, MTT reagent was removed, and formazan crystals were solubilized in DMSO (200 μ L). Absorbance at 490 nm was measured, cell viability was calculated as:

$$\text{Viability} = [(OD_{ps} - OD_{blank}) / (OD_{control} - OD_{blank})] \times 100$$

where

- OD_{ps} represents the absorbance of the therapeutic group,
- OD_{blank} represents the absorbance of the blank control (no cells),
- $OD_{control}$ represents the absorbance of the control group.

4.10 | Tumor Suppression Experiments In Vivo

When tumors reached 85–100 mm³, SKOV3-bearing mice were randomly assigned to five groups ($n = 5/\text{group}$). The control groups received PBS (saline), Dox, or TBD (1 mM, 100 μ L), while treatment groups received cRBD or NBD (1 mM, 100 μ L). Mice were treated intravenously four times on days 12, 14, 16, and 18 post-inoculations. Tumor volumes and body weights were recorded twice a week from day 5 to day 46. Tumor volume was calculated using the standard formula:

$$[\text{Length (L)} \times \text{Width}^2 (\text{D}^2)] / 2$$

Where:

- **V** is the tumor volume,
- **L** is the longest diameter of the tumor,
- **D** is the perpendicular diameter to **L**.

4.11 | Statistics and Reproducibility

All experiments were performed independently at least twice with consistent results. Biologically independent samples or animals were used in each group. Statistical analysis was performed using a one-tailed Student's t-test, with significance set at $p < 0.05$. All image-based experiments were conducted with proper blinding, and no data were excluded from analysis.

Author Contributions

Sanu Karan: Conceptualization, methodology, investigation (specifically the design and synthesis of chemicals), validation (solution-phase testing), and writing – original draft. **Hyunseung Lee:** Investigation (development and execution of in vivo mouse study), Data curation, and formal analysis. **Rema Naskar:** Investigation (conducting in vitro experiments), validation, and formal analysis. **Mi Young Cho, Hye Sun Park, and Eun Hee Han:** Investigation (conducting in vitro experiments), and validation. **Soumyadip Hore:** Computational HOMO–LUMO analysis. **Sun Hee Cho:** Investigation of intracellular uptake (initial study). **Truc Ngoc Kim Lam:** Investigation (conducting in vitro experiments). **Tae-woong Son:** Investigation (execution of in vivo mouse study). **Tae Jung Park:** Validation of synthesized constructs. **Inkyu Hwang:** Validation of in vitro experimental results. **Jonathan L. Sessler:** Conceptual design

and planning of this study, supervision, resources, and writing – review & editing. **Kwan Soo Hong:** Supervision, project administration, funding acquisition, and writing – review & editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

1. F. S. Collins and H. Varmus, “A New Initiative on Precision Medicine,” *New England Journal of Medicine* 372 (2015): 793–795, <https://doi.org/10.1056/NEJMp1500523>.
2. E. A. Ashley, “Towards Precision Medicine,” *Nature Reviews Genetics* 17 (2016): 507–522, <https://doi.org/10.1038/nrg.2016.86>.
3. J. L. Jameson and D. L. Longo, “Precision Medicine—Personalized, Problematic, and Promising,” *Obstetrical & Gynecological Survey* 70 (2015): 612–614, <https://doi.org/10.1097/01.ogx.0000472121.21647.38>.
4. L. Ma, Y. Shan, R. Bai, et al., “A Therapeutically Targetable Mechanism of BCR-ABL-Independent Imatinib Resistance in Chronic Myeloid Leukemia,” *Science Translational Medicine* 6 (2014): 252ra121.
5. L. Marques, B. Costa, M. Pereira, et al., “Advancing Precision Medicine: A Review of Innovative In Silico Approaches for Drug Development, Clinical Pharmacology and Personalized Healthcare,” *Pharmaceutics* 16 (2024): 332, <https://doi.org/10.3390/pharmaceutics16030332>.
6. C. Y. Lu, V. Terry, and D. M. Thomas, “Precision Medicine: Affording the Successes of Science,” *npj Precision Oncology* 7 (2023): 3, <https://doi.org/10.1038/s41698-022-00343-y>.
7. B. J. Burkett, D. J. Bartlett, P. W. McGarrah, et al., “A Review of Theranostics: Perspectives on Emerging Approaches and Clinical Advancements,” *Radiology Imaging Cancer* 5 (2023): 2201571.
8. T. H. Kim, S. Lee, and X. Chen, “Nanotheranostics for Personalized Medicine,” *Expert Review of Molecular Diagnostics* 13 (2013): 257–269, <https://doi.org/10.1586/erm.13.15>.
9. D. Ho, S. R. Quake, E. R. McCabe, et al., “Enabling Technologies for Personalized and Precision Medicine,” *Trends in Biotechnology* 38 (2020): 497–518, <https://doi.org/10.1016/j.tibtech.2019.12.021>.
10. S. Muyldermans, “Nanobodies: Natural Single-Domain Antibodies,” *Annual Review of Biochemistry* 82 (2013): 775–797, <https://doi.org/10.1146/annurev-biochem-063011-092449>.
11. E. Y. Yang and K. Shah, “Nanobodies: Next Generation of Cancer Diagnostics and Therapeutics,” *Frontiers in Oncology* 10 (2020): 1182.
12. L. Liu, B. Tu, Y. Sun, et al., “Nanobody-Based Drug Delivery Systems for Cancer Therapy,” *Journal of Controlled Release* 381 (2025): 113562, <https://doi.org/10.1016/j.jconrel.2025.02.058>.
13. S. Sun, Z. Ding, X. Yang, et al., “Nanobody: A Small Antibody With Big Implications for Tumor Therapeutic Strategy,” *International Journal of Nanomedicine* 16 (2021): 2337–2356, <https://doi.org/10.2147/IJN.S297631>.
14. S. Karan, J.-H. Cho, C. T. N. Tran, et al., “Activatable Near-Infrared Fluorescence And Chemical Exchange Saturation Transfer MRI Multimodal Imaging Probe for Tumor Detection In Vitro and In Vivo,” *Sensors*

- and *Actuators B: Chemical* 413 (2024): 135839, <https://doi.org/10.1016/j.snb.2024.135839>.
15. S. Karan, M. Y. Cho, H. Lee, et al., “Near-Infrared Fluorescent Probe Activated by Nitroreductase for In Vitro and In Vivo Hypoxic Tumor Detection,” *Journal of Medicinal Chemistry* 64 (2021): 2971–2981, <https://doi.org/10.1021/acs.jmedchem.0c02162>.
 16. M. Grossi, M. Morgunova, S. Cheung, et al., “Lysosome Triggered Near-Infrared Fluorescence Imaging of Cellular Trafficking Processes in Real Time,” *Nature Communications* 7 (2016): 10855, <https://doi.org/10.1038/ncomms10855>.
 17. Q. Wang, J. Guan, J. Wan, and Z. Li, “Disulfide Based Prodrugs for Cancer Therapy,” *RSC Advances* 10 (2020): 24397.
 18. J. Zhao, X. Li, T. Ma, B. Chang, B. Zhang, and J. Fang, “Glutathione-Triggered Prodrugs: Design Strategies, Potential Applications, and Perspectives,” *Medicinal Research Reviews* 44 (2024): 1013–1054, <https://doi.org/10.1002/med.22007>.
 19. W. Cai and X. Chen, “Anti-Angiogenic Cancer Therapy Based on Integrin $\alpha v \beta 3$ Antagonism,” *Anti-Cancer Agents in Medicinal Chemistry* 6 (2006): 407–428, <https://doi.org/10.2174/187152006778226530>.
 20. Z. Liu, F. Wang, and X. Chen, “Integrin $\alpha v \beta 3$ -Targeted Cancer Therapy,” *Drug Development Research* 69 (2008): 329–339, <https://doi.org/10.1002/ddr.20265>.
 21. D. Arosio, C. Casagrande, and L. Manzoni, “Integrin-Mediated Drug Delivery in Cancer and Cardiovascular Diseases With Peptide-Functionalized Nanoparticles,” *Current Medicinal Chemistry* 19 (2012): 3128–3151, <https://doi.org/10.2174/092986712800784748>.
 22. Z. Zhao, A. Ukidve, J. Kim, and S. Mitragotri, “Targeting Strategies for Tissue-Specific Drug Delivery,” *Cell* 181 (2020): 151–167, <https://doi.org/10.1016/j.cell.2020.02.001>.
 23. Y. Zhang, Y. Ji, Y. Tu, and Y. Li, “Autophagy in Doxorubicin Resistance: Basic Concepts, Therapeutic Perspectives and Clinical Translation,” *Frontiers in Immunology* 16 (2025): 1642050, <https://doi.org/10.3389/fimmu.2025.1642050>.
 24. J. Chen, Y. Shen, B. Wu, et al., “CUR5g, a Novel Autophagy Inhibitor, Exhibits Potent Synergistic Anticancer Effects With Cisplatin Against Non-Small-Cell Lung Cancer,” *Cell Death Discovery* 8 (2022): 435, <https://doi.org/10.1038/s41420-022-01217-9>.
 25. K. Chang, K. S. Hong, and C.-W. Kim, Single Domain Antibody Targeting $\alpha v \beta 3$ Integrin (Korea Patent, 2020), 10–2098462.
 26. S. Muyldermans, “Nanobodies: Natural Single-Domain Antibodies,” *Annual Review of Biochemistry* 82 (2013): 775–797, <https://doi.org/10.1146/annurev-biochem-063011-092449>.
 27. W. R. Strohl, “Current Progress in Innovative Engineered Antibodies,” *Protein & Cell* 9 (2018): 86–120, <https://doi.org/10.1007/s13238-017-0457-8>.
 28. I. Jovčevska and S. Muyldermans, “The Therapeutic Potential of Nanobodies,” *Biodrugs* 34 (2020): 11–26, <https://doi.org/10.1007/s40259-019-00392-z>.
 29. H. Bakherad, F. Ghasemi, M. Hosseindokht, and H. Zare, “Nanobodies; New Molecular Instruments With Special Specifications for Targeting, Diagnosis and Treatment of Triple-Negative Breast Cancer,” *Cancer Cell International* 22 (2022): 245, <https://doi.org/10.1186/s12935-022-02665-0>.
 30. M. H. Abdolvahab, P. Karimi, N. Mohajeri, M. Abedini, and H. Zare, “Targeted Drug Delivery Using Nanobodies to Deliver Effective Molecules to Breast Cancer Cells: The Most Attractive Application of Nanobodies,” *Cancer Cell International* 24 (2024): 67, <https://doi.org/10.1186/s12935-024-03259-8>.
 31. R. G. Parr and Y. Weitao, (1995), <https://doi.org/10.1093/oso/9780195092769.001.0001>.
 32. M. J. Frisch, *Gaussian 09 Revision A.02* (Gaussian, Inc., 2016).
 33. C. Lee, W. Yang, and R. G. Parr, “Development of the Colle-Salvetti Correlation-Energy Formula Into a Functional of the Electron Density,” *Physical Review B* 37 (1988): 785–789, <https://doi.org/10.1103/PhysRevB.37.785>.
 34. A. D. Becke, “A New Mixing of Hartree–Fock and Local Density-Functional Theories,” *The Journal of Chemical Physics* 98 (1993): 1372–1377, <https://doi.org/10.1063/1.464304>.
 35. S. Grimme, J. Antony, S. Ehrlich, and H. Krieg, “A Consistent and Accurate Ab Initio Parametrization of Density Functional Dispersion Correction (DFT-D) for the 94 Elements H–Pu,” *The Journal of Chemical Physics* 132 (2010): 154104, <https://doi.org/10.1063/1.3382344>.
 36. R. Ditchfield, W. J. Hehre, and J. A. Pople, “Self-Consistent Molecular-Orbital Methods. IX. An Extended Gaussian-Type Basis for Molecular-Orbital Studies of Organic Molecules,” *The Journal of Chemical Physics* 54 (1971): 724–728, <https://doi.org/10.1063/1.1674902>.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting File: adhm71149-sup-0001-SuppMat.docx.