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ORIGINAL ARTICLE

Monomethyl auristatin E-conjugated anti-EGFR antibody inhibits the growth of human EGFR-positive non-small cell lung cancer

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Abstract

Purpose Epidermal growth factor receptor (EGFR) is highly expressed on non-small cell lung cancers (NSCLC) and a valuable therapeutic target. This study aimed at producing and characterizing monomethyl auristatin E (MMAE)-conjugated anti-EGFR antibody as a novel EGFR-targeting therapy for NSCLC.

Methods A humanized anti-EGFR monoclonal antibody (named RC68) was purified and conjugated with MMAE using a MC-VC-PAB or PY-VC-PAB linker. The in vitro and in vivo antitumor activity of RC68-MC-VC-PAB-MMAE and RC68-PY-VC-PAB-MMAE were characterized.

Results The RC68 was generated from RC68-expressing cells and had a purity of > 99.0%. The RC68 recognized EGFR on tumor cells, particularly for higher EGFR expressing H125, A431, HCC827 and H1975 cells. The RC68 was conjugated with an average of 4 MMAE molecules to generate RC68-MC-VC-PAB-MMAE and RC68-PY-VC-PAB-MMAE, respectively. The RC68-MC-VC-PAB-MMAE, RC68-PY-VC-PAB-MMAE and RC68 displayed similar binding affinity to EGFR on tumor cells, and RC68-MC-VC-PAB-MMAE and RC68-PY-VC-PAB-MMAE were effectively internalized by H125 cells. The RC68-MC-VC-PAB-MMAE and RC68-PY-VC-PAB-MMAE inhibited the growth of H125 cells in vitro with an IC_{50} 7.37–8.04 ng/mL and implanted H125 tumors in vivo, but did not affect body weights of mice. The antitumor effect of RC68-MC-VC-PAB-MMAE was stronger than RC68-PY-VC-PAB-MMAE, which was also stronger than docetaxel in vivo.

Conclusions These novel antibody–drug conjugates, particularly for RC68-MC-VC-PAB-MMAE, may be a potential candidate for treatment of EGFR + NSCLC.

Keywords Epidermal growth factor receptor · Non-small cell lung cancer · Monoclonal antibody · Antibody–drug conjugates

Introduction

Non-small cell lung cancer (NSCLC) is still the leading cause of cancer-related mortality although treatments of NSCLC have greatly advanced [1]. NSCLC accounts for 85% of all lung cancers [2] and its 5-year survival rate remains low [3]. Due to its high mortality and incidence, developing novel and effective treatments for NSCLC are urgently needed. Currently, therapeutic strategies for early-stage NSCLC include surgical intervention, targeting therapies, chemotherapy, and radiation therapy, but all of them elicit undesirable side effects. The targeted therapies are interesting due to their specificity to cancer cells and minimal adverse effects [4]. A number of molecularly targeted therapies focus on various receptor tyrosine kinases (RTKs) involved in cellular growth and survival, and have been demonstrated to be effective. However, due to drug resistance, their therapeutic efficient duration is limited.

Epidermal growth factor receptor (EGFR) is a member of the RTK family, and is encoded by the proto-oncogene c-erb-B1 [5]. EGFR is usually present in an inactive form as a monomer. When engaged by its ligand, such as epidermal growth factor (EGF), the EGFR dimerizes and

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phosphorylates spontaneously, leading to activation of downstream signaling that regulates the growth, differentiation, and apoptosis of cells [6, 7]. EGFR is highly expressed in approximately 40–80% of NSCLC tumors and EGFR mutation is associated spontaneous activation of downstream signaling and is commonly detected in NSCLC tumors. The mutated EGFR naturally promotes the growth, invasion, and tumor angiogenesis [8, 9] and is associated with poor prognosis [10]. Currently, targeting EGFR therapies are the most promising strategies for the treatment of NSCLC in the clinic [11].

Besides small molecule tyrosine kinase inhibitors (TKIs), anti-EGFR monoclonal antibodies (mAbs) have been approved for treatment of advanced NSCLC [12]. TKIs are widely used drugs for treatment of NSCLC [13] and they include erlotinib [14], gefitinib [15], afatinib [16] and osimertinib [17] and others. However, the acquired drug resistance limits the duration of their clinical benefit [18–20]. Anti-EGFR mAb can specifically recognize the extracellular domain of EGFR and block the binding of ligand, thus prevent EGFR activation [21]. Furthermore, anti-EGFR mAb can promote the endocytosis of EGFR and reduce its density on cell membrane, and trigger antibody-dependent cell-mediated cytotoxicity (ADCC) against EGFR+ tumor cells [22]. Currently, necitumumab is the only anti-EGFR approved for NSCLC by the US Food and Drug Administration (FDA). However, due to the limited efficacy of necitumumab alone, it needs to be used in combination with other chemotherapy [23].

Antibody–drug conjugates (ADCs) are potent cytotoxic agents by linking cytotoxic small molecules to Abs that directly recognize a specific antigen on tumor cell surface [24]. Once bound to targeting antigen on the surface of tumor cells, the ADCs can be internalized by the cells via endocytosis, and subsequently, the linker between Ab and active drug can be metabolized by lysosomal proteases to release the active drug that kills tumor cells. Currently, more than 65 ADCs are undergoing in different stages of clinical trials [25], but no EGFR-targeting ADC for treatment of cancer has been approved by the FDA. The depatuxizumab–mafodotin, also called ABT-414, is one EGFR-targeting ADC by conjugating a humanized EGFR mAb (ABT-806) with monomethyl auristatin F (MMAF), a tubulin inhibitor, via a non-cleavable linker. A previous study has reported that ABT-414 has potent antitumor effects on glioblastoma multiforme (GBM) and controllable ocular toxicity in phase II clinical trials [26].

The EGFR overexpression on the surface of NSCLC tumor cells and its ability to be endocytosed make EGFR an idea target of ADCs [27]. The mAb, linker and cytotoxin are crucial for the efficacy and toxicity of an ADC. In addition, the coupling strategy is one critical factor for the design and development of ADCs for enhancing their overall functionality. In this study, we conjugated a high-toxic antimitotic drug monomethyl auristatin E (MMAE) to a humanized anti-EGFR mAb (called RC68) using cleavable MC-VC-PAB and PY-VC-PAB linkers to generate RC68-MC-VC-PAB-MMAE and RC68-PY-VC-PAB-MMAE ADCs. Subsequently, we characterized and investigated their chemical characteristics and in vitro and in vivo antitumor activities.

Materials and methods

Reagents

MC-VC-PAB-MMAE and PY-VC-PAB-MMAE were synthesized in our laboratory. Other reagents included Protein A and Sepharose High Performance resin (GE Healthcare, Uppsala, Sweden), Cell Counting Kit-8 (CCK-8, DOJINDO, Kumamoto, Japan), pHAb Amine Reactive Dyes (Promega, Madison, WI, USA), Fluorescein (FITC)-AffiniPure goat anti-human IgG (Fcγ fragment specific, Jackson Immunoresearch Laboratories, West Grove, PA, USA), docetaxel (Sanofi, Paris, France), soluble extracellular domain of EGFR (EGFR-ECD, G&P Biosciences, Santa Clara, CA, USA), and cetuximab (Merck KGaA, Darmstadt, Germany) as well as other chemicals and reagents (Sigma-Aldrich, St. Louis, USA).

Cell lines and culture

Human embryonic kidney (HEK)-293, human squamous carcinoma cell A431, and non-small cell lung cancer HCC827, H1975, NCI-H460 and H125 cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in RM1640 medium supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified incubator with 5% CO_2. RC68 expressing CHO cell line was generated and stored in our laboratory [28].

Animals

Female BALB/c nude mice (18–22 g, 5–7 weeks old) were obtained from CRL (Beijing, China). The mice were housed in a specific pathogen-free facility with free access to autoclaved food and water in the Animal Research Center of Medicilon (Shanghai, China).

Preparation of humanized anti-EGFR antibody RC68

Humanized anti-EGFR antibody RC68 was prepared, as our previous description [28]. Briefly, RC68 expressing CHO cells were cultured in a STR50L bioreactor (Sartorius Stedim, Guxhagen, Germany) by fed batch process developed

for the cell line. After 15 days of cultivation, the culture broth was clarified and RC68 was purified by the protein A affinity chromatography and Sepharose high performance chromatography. The purity of RC68 was analyzed by hydrophobic interaction chromatography–high performance liquid chromatography (HIC–HPLC). The concentrations of RC68 were determined by UV absorption at 280 nm.

Flow cytometry

The levels of EGFR expression in A431, HCC827, H1975, H125, NCI-H460, and HEK293 cells were examined by flow cytometry after immunofluorescent staining. Briefly, the cells (1×10^5 cells/tube) were incubated in duplicate with 0.5 μg RC68 (5 μg/mL) or IgG1 at 4 °C for 20 min. After being washed, the cells were stained with FITC-AffiniPure goat anti-human IgG (Fcγ fragment specific; 15 μg/mL) at 4 °C for 20 min. The fluorescent signals in individual samples were analyzed by flow cytometry on an Accuri C6 Plus flow cytometer (BD Bioscience, USA) using the FlowJo software.

Preparation of ADCs

Two ADCs were prepared using MC-VC-PAB and PY-VC-PAB linkers that could be cleaved by intracellular proteases, respectively [29]. MMAE was conjugated to the antibody through MC-VC-PAB using the conventional sulfhydryl coupling method [29]. In this way, MMAE was attached to thiol groups of antibody chains through a linking moiety. PY-VC-PAB was designed as a linker that covalently connected to thiol groups with a coupling agent to make the thiol bridges linking two heavy chains or the heavy with light chains in a given antibody [30]. The drug–antibody ratios (DAR) of RC68-MC-VC-PAB-MMAE and RC68-PY-VC-PAB-MMAE ADCs were determined using size exclusion chromatography–mass spectrometry (SEC–MS) analysis [29].

Biacore for binding kinetics test of antibody and ADCs

The binding kinetics and affinities of RC68-based ADCs for soluble EGFR-ECD were measured using surface plasmon resonance (SPR) technology (Biacore T200, GE healthcare) [31]. Briefly, individual samples (10 nmol/L RC68, or RC68-MC-VC-PAB-MMAE, or RC68-PY-VC-PAB-MMAE, or cetuximab) in phosphate buffered saline (PBS, pH 7.4) were loaded on the Protein-A chip (GE healthcare) for 30 s. After being washed, the loaded samples on the chips were reacted with different concentrations (69.4, 34.7, 17.4, 8.68, and 4.34 nmol/L) of EGFR-ECD in PBS for 7500 s. During the reaction period, the kinetic association (K_a), disassociation (K_d) and affinities (K_D, $K_D = K_d/K_a$) were measured longitudinally using Biacore T200 evaluation 3.1 software (GE healthcare).

Examination of the internalization of ADCs

RC68 and RC68-based ADCs were conjugated with pHAb Amine Reactive dyes [32]. This enabled maximum fluorescent signals of the antibody under intracellular acidic conditions without any fluorescent signals in the extracellular environments. H125 and NCI-H460 cells (1×10^5 cells/well) were cultured in duplicate into 6-well plates and treated with 100 μL of medium containing 1 μg pHAb amine reactive dye-conjugated RC68 or RC68-based ADCs at 37 °C for various periods. The internalization of RC68 and RC68-based ADCs was analyzed longitudinally (0 h, 1.5 h, 7.5 h and 24 h) by flow cytometry.

Cytotoxicity assay

A431, HCC827, H1975, H125, NCI-H460 and HEK293 cells (5×10^3 cells/well) were cultured in 96-well plates, and 12 h later the cells were treated in triplicate with varying concentrations of ADCs or RC68 for 72 h. The cell viability was determined by the CCK-8 assay using a specific kit, according to the manufacturer's instructions. The cells cultured in medium alone served as the control. The absorbent optical density (OD) values at 450 nm were measured in a microplate reader (M5e, MD, USA). The inhibition rate of cell growth in individual wells was determined using the following formula: inhibition rate = (OD value of control (without ADCs or RC68 alone) − OD value of dose)/OD value of control × 100%. The IC_{50} values (concentration of inhibitor resulting in 50% inhibition) of the two types of ADCs for the cells were calculated using the Prism 6 software.

Xenograft in mice

BALB/c nude mice were injected subcutaneously (s.c.) with H125 cells (5×10^6 per mouse) in Matrigel to induce human NSCLC in mice. When the tumor volume reached 100–300 mm^3, the mice were randomized and treated intravenously with vehicle, different concentrations (2.5, 5 or 10 mg/kg body weights) of RC68-MC-VC-PAB-MMAE or RC68-PY-VC-PAB-MMAE, 10 mg/kg docetaxel (positive control) or RC68 (negative control) weekly for 4 weeks. Their body weights and tumor volumes were monitored twice per week. Tumor volumes were determined by the formula: tumor volume (mm^3) = long diameter × (short diameter)^2 × 0.5.

Statistical analysis

Data are expressed as mean ± standard deviation (SD) or mean ± standard error of the mean (SEM). The difference between groups was determined using the Student's *t* test and the difference among groups was determined by repeated ANOVA tests. Statistical analyses were performed using Graphpad Prism 6 software for Windows (Graphpad Software). A P value of < 0.05 was considered statistically significant.

Results

The produced RC68 recognizes EGFR on human NSCLC cells

RC68 is a humanized anti-EGFR IgG1 monoclonal antibody. To prepare RC68-based ADCs, RC68-expressing CHO cells were cultured in a STR50L bioreactor by fed batch cultivation and the RC68 was purified. As a result, 90 g RC68 was yielded. SDS-PAGE analysis revealed that RC68 in either reduction or non-reduction condition displayed molecular weights as expected, respectively [28]. HIC–HPLC analysis indicated that the purified RC68 antibody had a purity of > 99% (Fig. 1a). After indirectly stained with RC68 or control IgG1 and fluorescent second antibodies, the intensity of EGFR expression (Supplementary Fig. 1) and the frequency of EGFR+ cells (Fig. 1b) were determined by flow cytometry. Quantitative analysis revealed that the levels of EGFR expression in H125, A431, HCC827 and H1975 cells were significantly higher than that in NCI-H460 cells, and there was no detectable EGFR expression in HEK293 cells (Supplementary Fig. 1). Such data clearly demonstrated that the produced RC68 recognized EGFR on NSCLC cells and EGFR was highly expressed on H125, A431, HCC827 and H1975 cells. The produced RC68 provided a valuable resource for the generation of ADCs.

Preparation and characterization of RC68-based ADCs

RC68-based RC68-MC-VC-PAB-MMAE and RC68-PY-VC-PAB-MMAE ADCs were prepared by coupling cytotoxin MMAE to RC68 using intracellularly cleavable MC-VC-PAB or PY-VC-PAB linkers. Their structures are illustrated in Fig. 2a. Further analysis of the DAR of RC68-based ADCs by SEC–MS revealed that the RC68-MC-VC-PAB-MMAE exhibited 2, 4 and 6 drug molecules per antibody at three major peaks, while RC68-PY-VC-PAB-MMAE conjugated 3, 4 and 5 drug molecules per antibody (Fig. 2b). The majority of RC68-based ADCs conjugated four MMAE molecules in our experimental conditions.

EGFR-binding affinity of RC68-based ADCs

Next, the EGFR-binding affinity of RC68-based ADCs or control RC68 and FDA-approved cetuximab (Erbitux), a recombinant human/mouse chimeric mAb was analyzed by biacore technology (Fig. 3a). The K_a values of RC68, RC68-based ADCs and cetuximab for EGFR-ECD were similar, while the K_d values of RC68 and RC68-ADCs were much lower than cetuximab (Fig. 3b). There was no significant change in avidity with EGFR-ECD after conjugation of RC68, and the kinetic binding constant (K_D) of RC68 and ADCs ranged from 35.6 to 44.6 pM, which were obviously better than cetuximab with a K_D value of 1.02 nM (Fig. 3b). Hence, RC68-based ADCs had a high affinity to EGFR.

Internalization of RC68-based ADCs by NSCLC cells

To test whether RC68, RC68-MC-VC-PAB-MMAE, and RC68-PY-VC-PAB-MMAE could be internalized by NSCLC cells, H125 and NCI-H460 cells were treated with 1 μg pHAb amine reactive dye-conjugated RC68, RC68-MC-VC-PAB-MMAE, or RC68-PY-VC-PAB-MMAE up to 24 h. The intracellular fluorescent signals of individual groups of cells were measured longitudinally by flow cytometry (Fig. 4). The fluorescent signals increased gradually with time in both types of cells. There was no significant difference in the frequency of cells with positively fluorescent signals among the dye-conjugated RC68, RC68-MC-VC-PAB-MMAE, or RC68-PY-VC-PAB-MMAE-treated cells and the percentages of H125 cells with positively fluorescent signals were higher than that in NCI-H460 cells. Thus, RC68, RC68-MC-VC-PAB-MMAE, and RC68-PY-VC-PAB-MMAE were effectively internalized by H125 and NCI-H460 cells.

In vitro cell cytotoxicity of RC68-based ADCs

The effect of RC68-based ADCs on the growth inhibition of H125, A431, HCC827, H1975, NCI-H460, and HEK293 cells was examined by CCK-8 assay. While there was no significant inhibition of RC68 on the growth of all types of cells, both RC68-MC-VC-PAB-MMAE and RC68-PY-VC-PAB-MMAE showed a significant inhibition on the growth of H125, A431, HCC827 and H1975 cells in a dose-dependent manner with an IC_{50} value from 2.23 to 9.30 ng/mL (Fig. 5a, b). However, there was no clear inhibition on the growth of NCI-H460 and HEK293 cells, and the inhibition was not significant on NCI-H460 cells with the inhibition rates > 10 μg/mL for both RC68-MC-VC-PAB-MMAE and RC68-PY-VC-PAB-MMAE (Fig. 5b). Both RC68-MC-VC-PAB-MMAE and RC68-PY-VC-PAB-MMAE had strong cytotoxicity against H125, A431, HCC827 and H1975 cells, but not to NCI-H460 and HEK293 cells, indicating that the

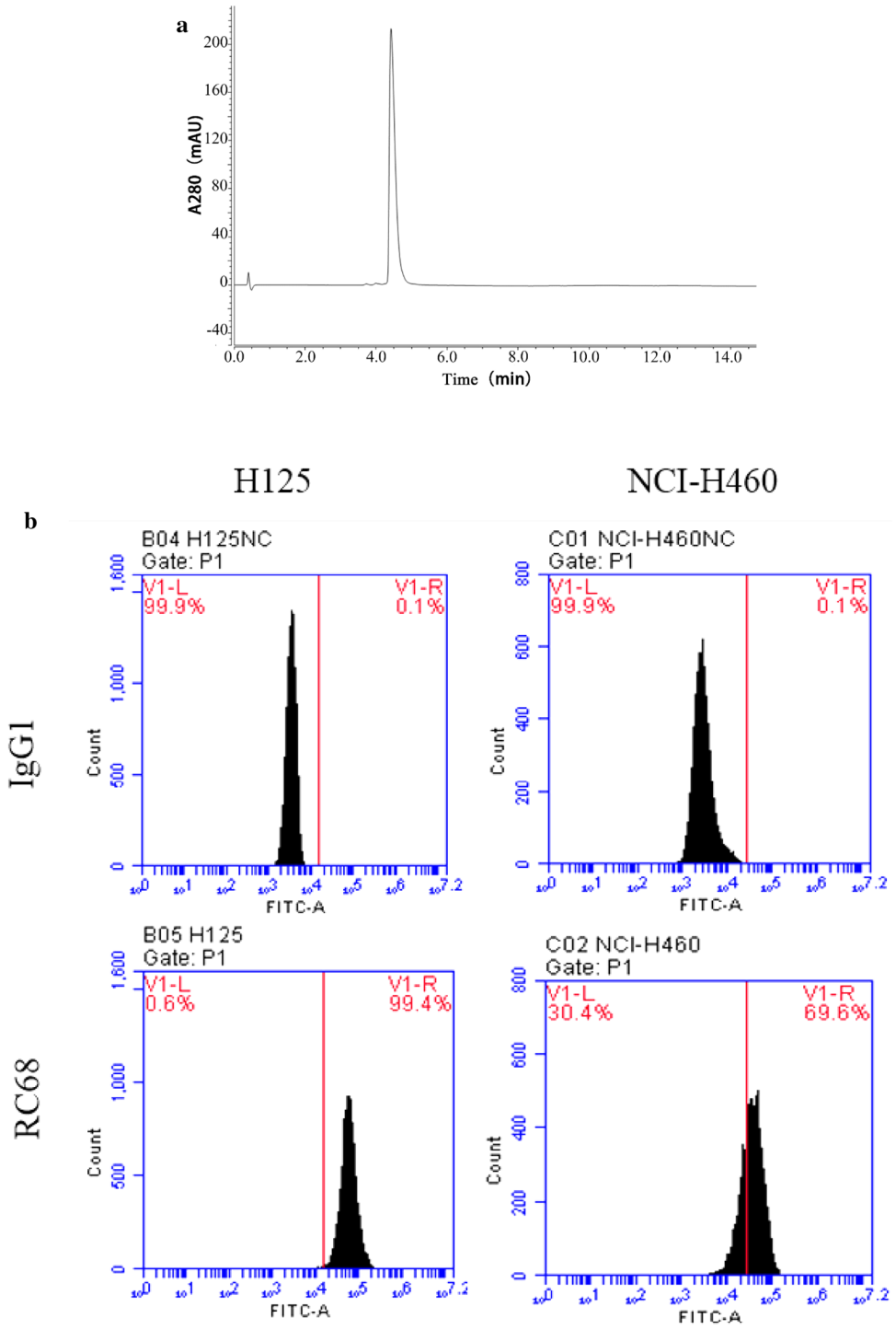


Fig. 1 Characterization of humanized anti-EGFR monoclonal antibody (RC68). **a** HIC–HPLC analysis of the purified RC68 with a purity of > 99%. **b** Quantitative analysis of fluorescent signals. H125 and NCI-H460 cells were treated with human IgG1 or RC68 and stained with FITC-AffiniPure goat anti-human IgG, followed by flow cytometry. Data are representative images/histograms of each group of cells from three separate experiments

cytotoxicity of RC68-based ADCs was dependent on the higher levels of EGFR expression on NSCLC cells.

In vivo antitumor efficacy of RC68-based ADCs

To determine therapeutic potential of RC68-based ADCs, BALB/c nude mice were injected with H125 cells to induce solid tumor and treated with the indicated doses of either drug weekly. We found that treatment with 10 mg/kg RC68-MC-VC-PAB-MMAE ($P < 0.001$), RC68-PY-VC-PAB-MMAE ($P < 0.001$), or docetaxel ($P < 0.001$), but not other reagent or doses, significantly decreased the H125 tumor volumes in mice and inhibitory effect of 10 mg/kg RC68-MC-VC-PAB-MMAE was significantly stronger than that of 10 mg/kg RC68-PY-VC-PAB-MMAE ($P < 0.01$) or 10 mg/kg docetaxel ($P < 0.01$, Fig. 6a, b).

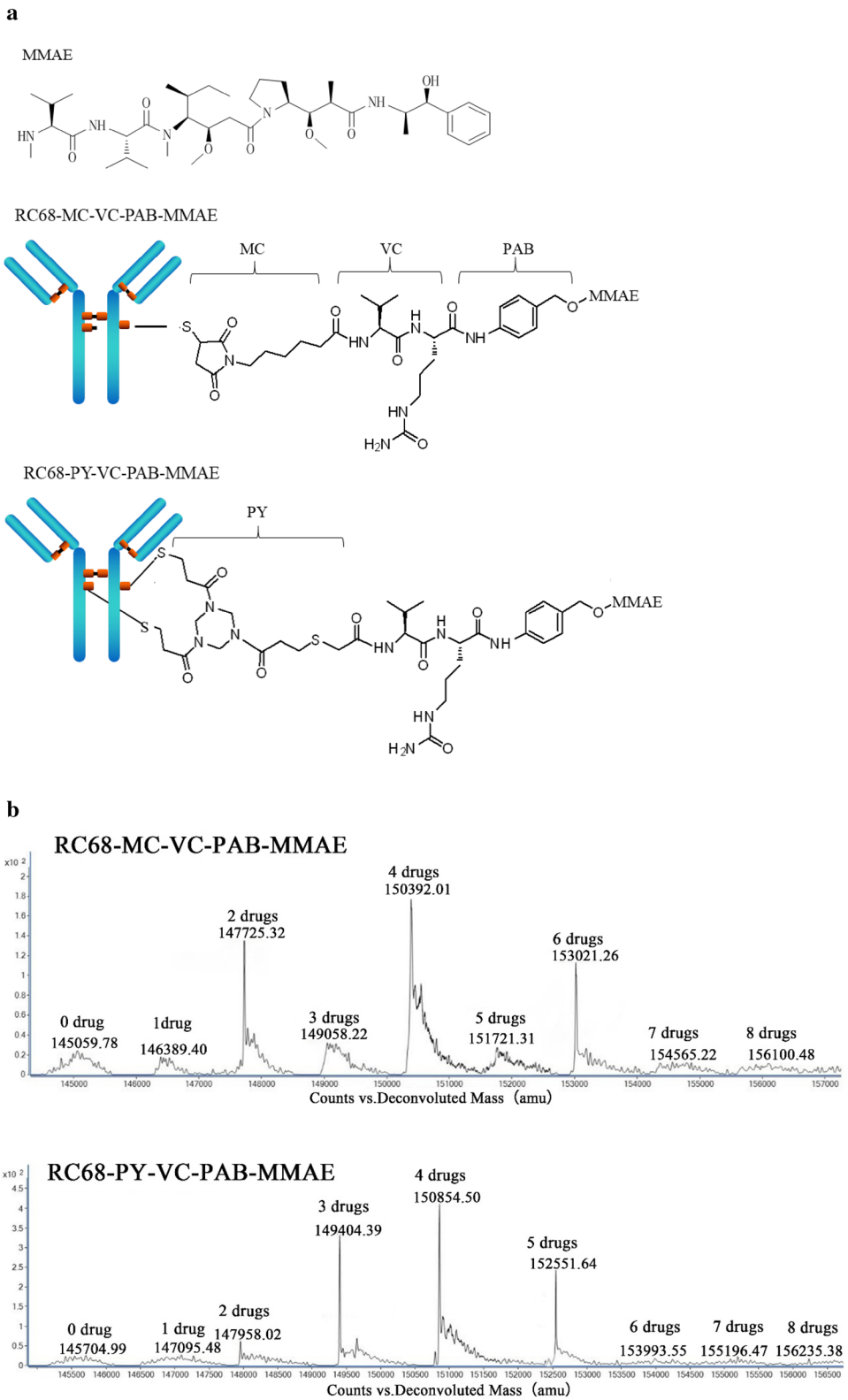
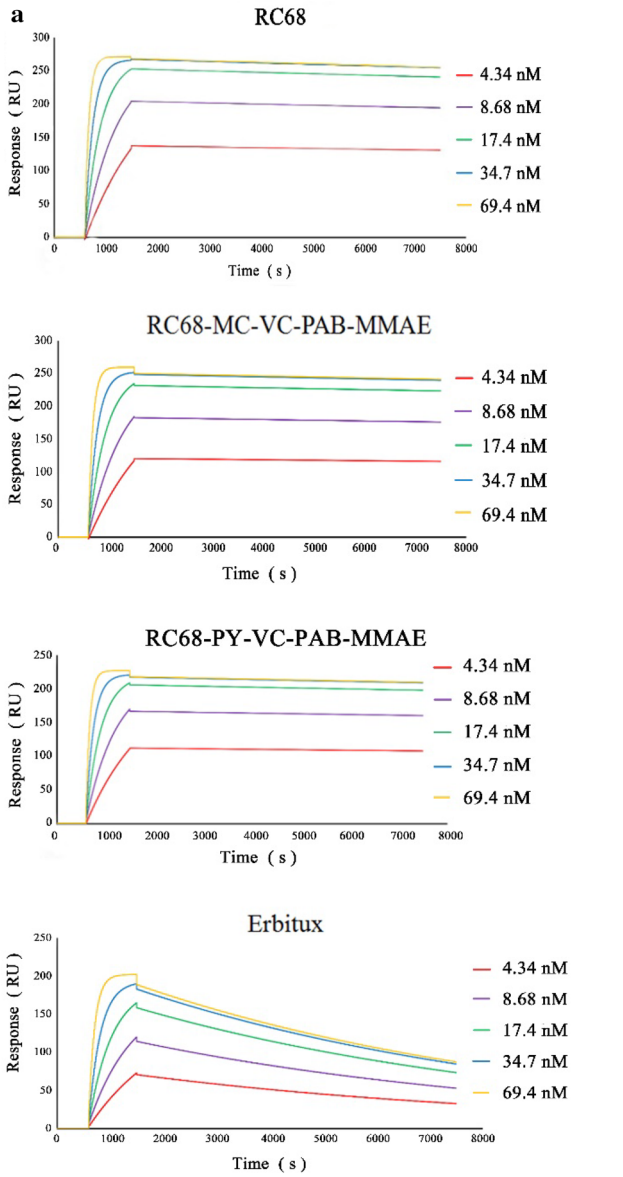


Fig. 2 Characterization of RC68-based ADCs. **a** Molecular structures of RC68-based ADCs. **b** The drug–antibody ratios (DAR) of RC68-based ADCs were analyzed by size exclusion chromatography–mass spectrometry (SEC–MS). Data are representative histograms from two separate experiments

Measurement of body weights indicated that treatment with 10 mg/kg docetaxel, but not another reagent, significantly reduced the body weights in mice (Fig. 6c). Therefore, treatment with 10 mg RC68-MC-VC-PAB-MMAE or RC68-PY-VC-PAB-MMAE significantly inhibited the growth of H125 tumors in mice, but did not affect their body weights.



b

Ligand	K_a ($M^{-1} \cdot S^{-1}$)	K_d (S^{-1})	K_D (M)
RC68	1.84×10^6	8.22×10^{-5}	4.46×10^{-11}
RC68-MC-VC-PAB-MMAE	1.68×10^6	6.09×10^{-5}	3.62×10^{-11}
RC68-PY-VC-PAB-MMAE	1.86×10^6	6.60×10^{-5}	3.56×10^{-11}
Erbitux	1.26×10^6	1.28×10^{-3}	1.02×10^{-9}

Fig. 3 Dynamic binding analysis of RC68-based ADCs. **a** The dynamic binding and dissociation curves. **b** The values of K_a, K_d and K_D were calculated using Biacore T200 software. The data are summarized as mean values of different concentrations of each drug

Discussion

EGFR-targeting therapies, such as EGFR-TKIs and EGFR-Abs, are effective strategy for treatment of NSCLC [33]. However, those therapeutic strategies do not directly kill tumor cells [33]. Furthermore, EGFR-targeted therapies usually have little effect on the KRAS-mutated advanced and/or metastatic NSCLC [34] and there is no effective therapy for control of KRAS mutation-related progression of NSCLC. Interestingly, EGFR-targeting ADCs can directly kill tumor cells after endocytosis, regardless of KRAS mutations, which may be a promising strategy for treatment of NSCLC.

In the present study, we generated and purified RC68, a humanized anti-EGFR IgG1 monoclonal antibody. We found that RC68 had a high affinity for binding to EGFR on NSCLC cells and was internalized by NSCLC cells in vitro. In our study, both RC68-MC-VC-PAB-MMAE and RC68-PY-VC-PAB-MMAE contained a protease-sensitive valine–citrulline (VC) dipeptide sequence that was designed for optimal stability in human plasma and efficient cleavage by human cathepsin B [29]. After internalization, both the mAb and linkers of these ADCs were metabolized by lysosomal proteases to release the active drug. The PY linker of RC68-PY-VC-PAB-MMAE can covalently reconnect the light and heavy chains as well as two heavy chains after the reduction of RC68. In contrast, the MC linker of RC68-MC-VC-PAB-MMAE can only be coupled to the thiol group of RC68, but cannot form covalent bonds between the two chains of the mAb, thus making the ADC more easily to be metabolized in the cells in vivo. Actually, both RC68-MC-VC-PAB-MMAE and RC68-PY-VC-PAB-MMAE had a similar DAR value and each ADC molecule contained approximately four MMAE molecules, indicating homogeneity of both ADCs. These findings support the notion that cysteine-based conjugation is superior to lysine-based conjugation to control DAR given the limited number of conjugation sites and the distinct reactivity of the thiol group in an IgG molecule [35, 36]. We chose to use MMAE because the commonly used MMAF and DM4 are associated with microcystic keratopathy and ocular side effect while the MMAE-based ADCs do not show corneal side effects [26, 37–39]. More importantly, we found that RC68 and RC68-based ADCs had similar affinity binding to EGFR+ NSCLC and were effectively internalized by EGFR+ NSCLC, particularly by high EGFR-expressing NSCLC cells. Given that the endocytosis of ADCs is critical for releasing active drug/cytotoxin and their cytotoxicity of ADCs [40, 41], the generated RC68-MC-VC-PAB-MMAE and RC68-PY-VC-PAB-MMAE may be valuable for potential safe therapies.

Actually, treatment with RC68 did not inhibit the growth of NSCLC cells in vitro and tumors in vivo,

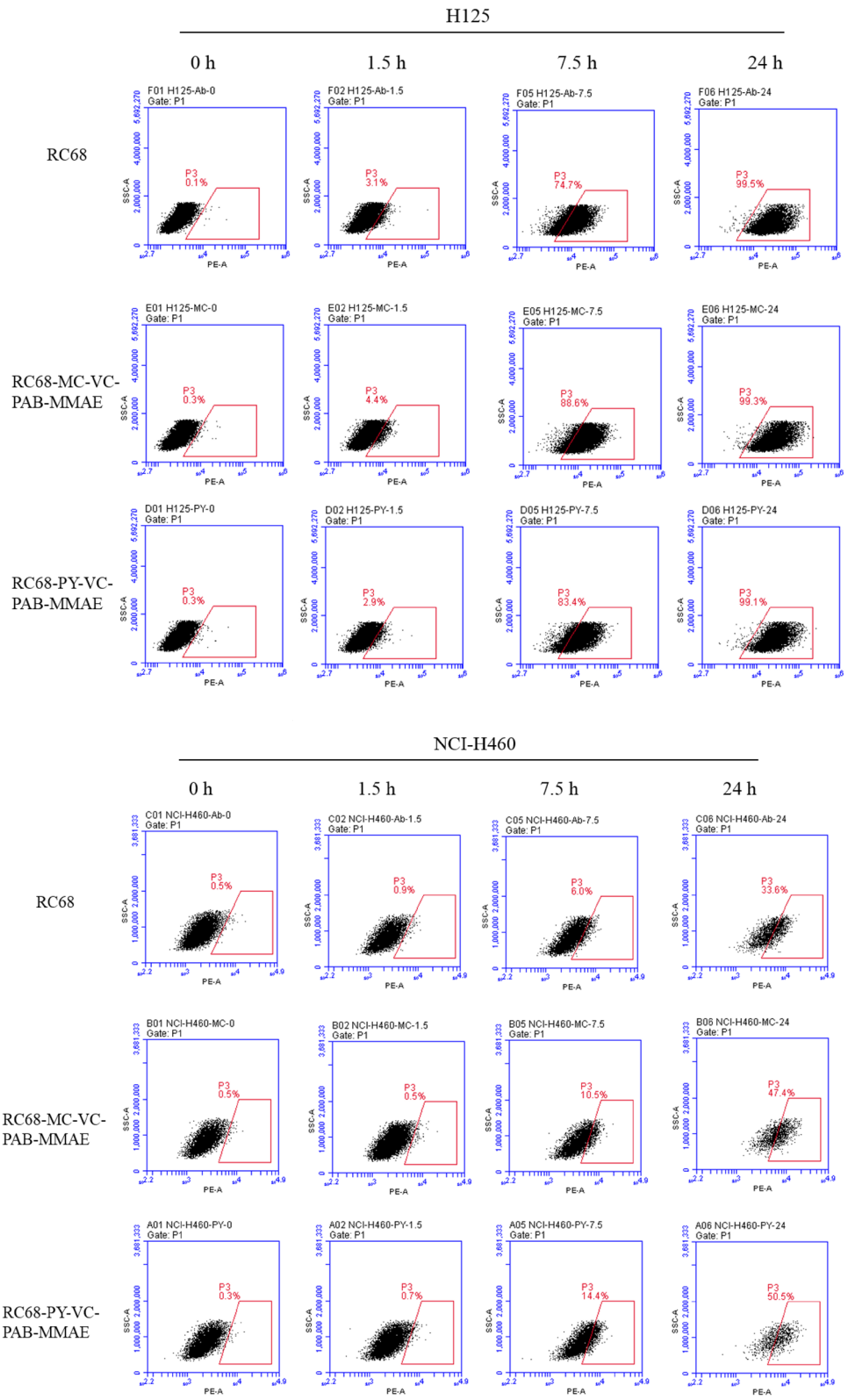
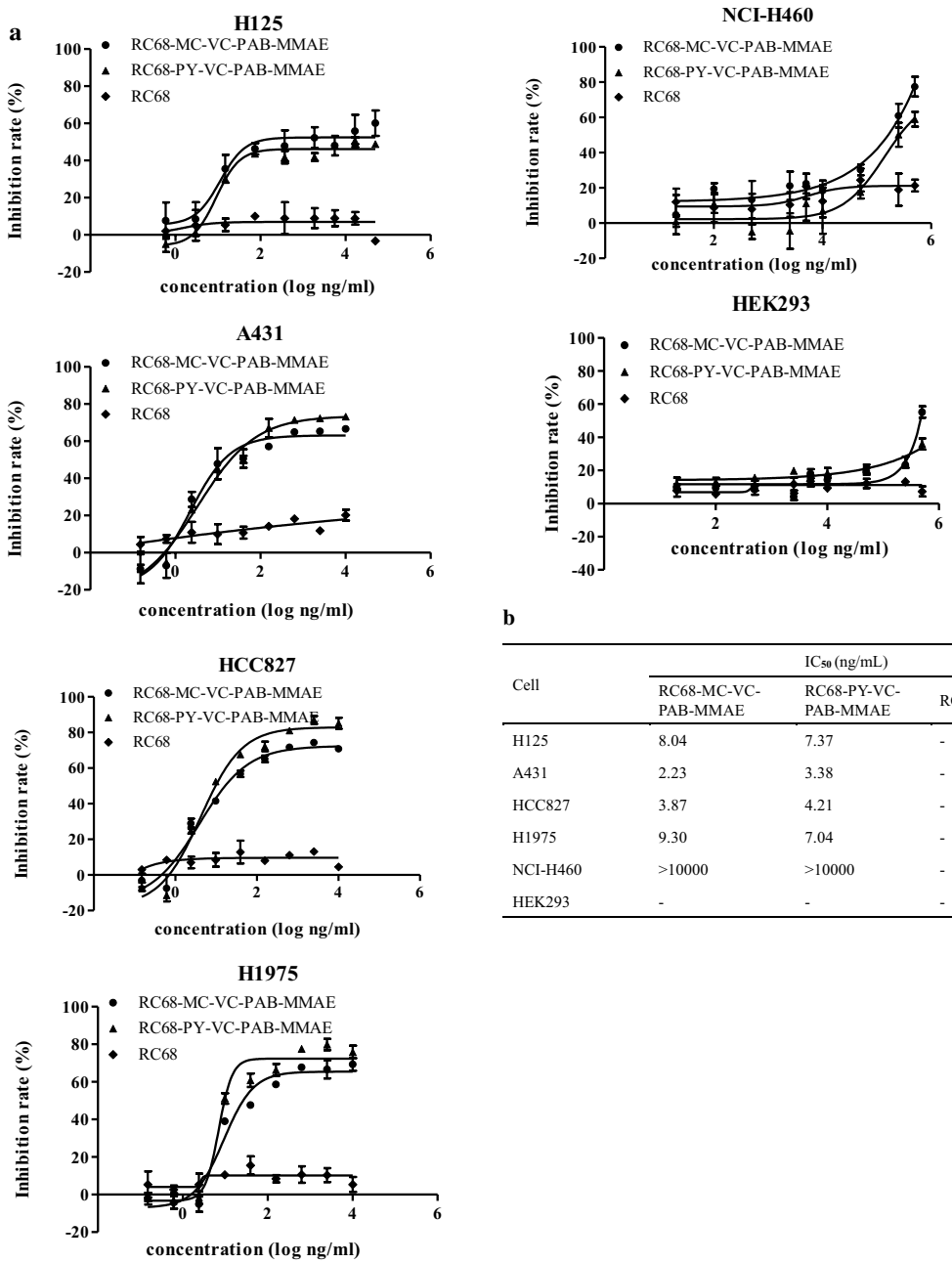


Fig. 4 Flow cytometry analysis of endocytosis of RC68-based ADCs. The endocytosis efficiency of RC68 and RC68-based ADCs in the indicated NSCLC cells was examined longitudinally by flow cytometry after stained with immunofluorescent-labeled each drug. Data are representative charts from two separate experiments

suggesting that the released toxin molecule, but not RC68, may be responsible for cytotoxicity against NSCLC. In this study, we found that treatment with either RC68-based ADC significantly inhibited the growth of high EGFR-expressing H125 cells in vitro and tumors in vivo, but not lower EGFR expressing NCI-H460 cells in vitro. Such



b

Cell	IC_{50} (ng/mL)		
	RC68-MC-VC-PAB-MMAE	RC68-PY-VC-PAB-MMAE	RC68
H125	8.04	7.37	-
A431	2.23	3.38	-
HCC827	3.87	4.21	-
H1975	9.30	7.04	-
NCI-H460	>10000	>10000	-
HEK293	-	-	-

Fig. 5 In vitro cytotoxicity of RC68-based ADCs. **a** The cytotoxicity of ADCs against H125, A431, HCC827, H1975, NCI-H460 and HEK293 cells was assessed by CCK-8 assays. The cells were treated with indicated doses of each drug for 72 h and exposed to CCK-8 reagent for 3 h. The OD values were measured in a microplate reader and the inhibition rates and IC_{50} values of each drug were calculated. **b** The IC_{50} values. Data are expressed as the mean ± SD or mean values from three separate experiments

data indicate that the antitumor effect of RC68-based ADCs depends on high levels of EGFR expression on the targeted NSCLC tumors. The resistance of NCI-H460 cells to ADCs may be not only from their lower levels of EGFR expression. Because the activity of ADCs depends on the efficient endocytosis and enzymatically metabolizing these ADCs to release cytotoxic compound intracellularly, the resistance of NCI-H460 cells may be also from lower efficacy in endocytosis and enzymatic metabolisms of ADCs in the cells. Furthermore, genetic and structural alteration in EGFR structure may also affect the binding, endocytosis and enzymatic metabolism of ADCs, leading to alteration in cytotoxicity of these ADCs. We are interested in further investigating the potential mechanisms underlying the resistance to ADCs in NSCLC cells. Our data extended previous observations that EGFR-targeting

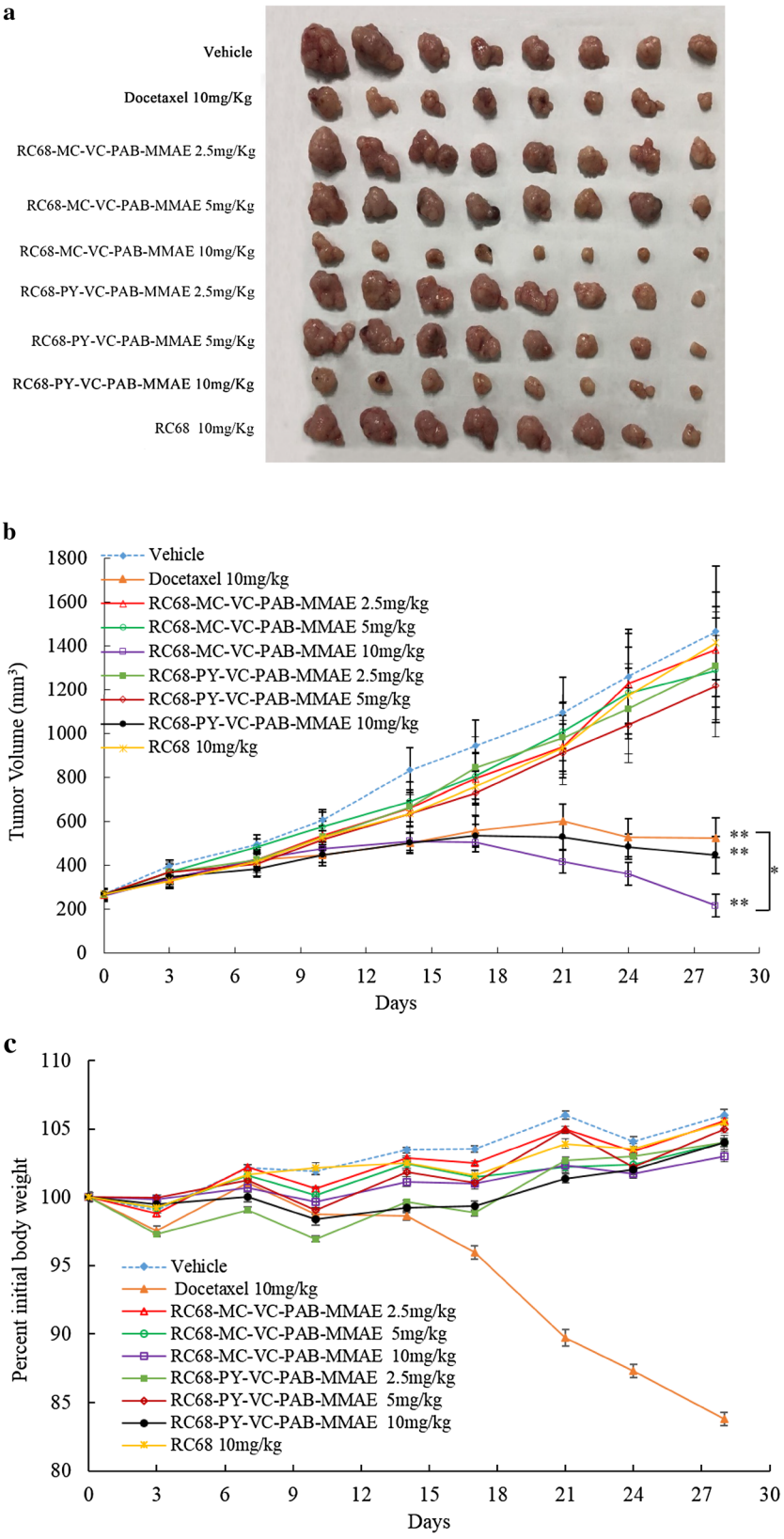


Fig. 6 In vivo antitumor activity of RC68-based ADCs. BALB/c nude mice were implanted subcutaneously with H125 cells and when the solid tumor reached 100–300 mm^3, the mice were randomized and treated intravenously with indicated drug weekly. The effect of each treatment on the growth of tumors was measured for tumor volumes and their body weights were measured twice per week. At the end of the experiment, the tumors were dissected and photoimaged. Data are representative image or expressed as the mean ± SEM of each group of mice ($n = 8$ per group). **a** The grown tumors; **b** dynamic growth of tumors; **c** the body weights of mice. $^*P < 0.01$, $^{**}P < 0.001$ vs. the vehicle group

ADCs can control the growth of GBM [26, 42]. More interestingly, the therapeutic effect of RC68-based ADCs in vivo was better than that of docetaxel in our experimental system. Treatment with either RC68-based ADC, unlike docetaxel, did not affect the body weights of mice, suggesting that these ADCs may be relatively safe in rodent in the experimental doses. Therefore, such findings are important for design of clinical trials for safe intervention of NSCLC and suggest that RC68-based ADCs may be valuable for other high EGFR-expressing malignant tumors.

In conclusion, we generated EGFR-targeting RC68-MC-VC-PAB-MMAE and RC68-PY-VC-PAB-MMAE with a cleavable linker. These ADCs, similar to RC68, had similar affinity binding to EGFR on NSCLC cells and were effectively internalized by EGFR + NSCLC cells. Furthermore, treatment with either ADC, but not RC68, effectively inhibited the growth of high EGFR-expressing H125 NSCLC cells in vitro and tumors in vivo without affecting body weights of mice. Such findings suggest that RC68-based ADCs, particularly for RC68-MC-VC-PAB-MMAE, may be promising therapeutic drugs for safe treatment of high EGFR-expressing NSCLC and other malignancies.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

Ethical approval Animal experiment was approved by Medicilon and carried out in accordance with the institutional guidelines. This study did not contain any experiment with human participants.

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