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PRECLINICAL STUDIES

Preclinical studies of targeted therapies for CD20-positive B lymphoid malignancies by Ofatumumab conjugated with auristatin

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Summary Utilization of antibodies to deliver highly potent cytotoxic agents to corresponding antigen-overexpressed tumor cells is a clinically validated therapeutic strategy. Ofatu mumab (OFA, trade name Arzerra) is a fully human CD20-specific antibody that is active against CD20-positive B-cell lymphoma/chronic lymphocytic leukemia cells. In order to further enhance the anticancer effect of OFA, anti-CD20 OFA has been conjugated with highly cytotoxic monomethyl auristatin E (MMAE) through a cathepsin-B-cleavable valine-citrulline (vc) dipeptide linkage to form OFA-vcMMAE and the anti-tumor activity of OFA-vcMMAE against CD20-positive B lymphoma cells are then evaluated in vitro and in vivo. As a result, conjugation of OFA with MMAE has kept the initial effector functional activities of OFA such as binding affinity, complement-dependent cytotoxicity (CDC) as well as antibody-dependent cell-mediated cytotoxicity (ADCC). In addition, the conjugation of MMAE significantly improved the cytotoxic activity of OFA against CD20-positive cells (i.e., Raji, Daudi and WIL2-S cells) but not against CD20-negative K562 cells. On the other hand, OFA-vcMMAE was modulated from the CD20-positive cell surface and then entered the lysosomes by receptor-mediated endocytosis, underwent proteolytic degradation and released active drug MMAE to induce apoptotic cell death through a caspase-3-like protease-dependent pathway. Surprisingly, OFA-vcMMAE completely inhibited the growth of CD20-positive Daudi and Ramos lymphoma xenografts in vivo, and exhibited greater anti-tumor activity than unconjugated OFA, suggesting that the anti-tumor activity of anti-CD20 antibody can be enhanced by conjugation with MMAE. In the near future, this new approach might be used as a clinical treatment of CD20-positive B lymphoid malignancies.

Keywords Ofatumumab · MMAE · Conjugate · CD20 · Targeted therapy

Abbreviations

Ab	Antibody
ADC	Antibody-drug conjugate
ADCC	Antibody-dependent cell-mediated cytotoxicity
CCK-8	Cell Counting Kit-8
CDC	Complement-dependent cytotoxicity
MAb	Monoclonal antibody
MFI	Geometric mean fluorescence intensity ratio
MMAE	Monomethyl auristatin E
OFA	Ofatumumab
PARP	Poly (ADP-ribose) polymerase
PI	Propidium iodide
vc	Valine-citrulline

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Introduction

Small-molecule anti-tumor drugs are widely used for cancer treatment in clinical practice. However, the non-target cell killing (i.e., both normal and cancer cells) by its small-molecular drugs

results in obvious limitations of their further applications and developments [1]. On the other hand, although the antibodies are able to bind to their corresponding antigen on tumor cells specifically, their therapeutic effects are often unsatisfactory. Antibody-drug conjugates (ADCs), a new sort of targeted medicine, usually use monoclonal antibodies (mAbs) to selectively deliver high-cytotoxic drugs to antigen-positive cancer cells so as to increase their cytotoxicity in tumor cells [2]. Likewise, ADCs can also effectively increase the cytotoxic drug concentrations at tumor sites and decrease systemic drug exposure to reduce side effects [3].

Since the U.S. FDA approved the first ADC, a humanized anti-CD33 conjugated to calicheamicin (gemtuzumab ozoga micin; Mylotarg), to treat acute myeloid leukemia in 2000, many ADCs have been actively pursued to combat different types of cancer. Now more than 20 ADCs are evaluated in various phases of clinical trials [4]. Excitingly, two novel ADCs, brentuximab vedotin (anti-CD30 Ab conjugated with MMAE) and T-DM1 (anti-HER2 Ab Herceptin conjugated with DM1) were respectively approved by FDA in 2011 and 2013 for the treatment of CD30-positive malignancies and HER2-positive breast cancer, indicating that the biotherapeutic drugs will be widely accepted for cancer treatments in the near future.

Generally, CD20 is a B-cell specific surface protein expressed on mature B lymphocytes. However, many reports have also found that CD20 is highly expressed on the cell membrane of most of the B-lymphocytic lymphomas, and this characteristic makes the CD20 antigen an appealing target for monoclonal antibody (mAb) therapy [5, 6]. Considerable efficacy of several anti-CD20 mAbs in the treatment of B-cell lymphomas has been achieved. For instance, Rituxan (Chimeric Antibody) was the first anti-CD20 mAb approved by FDA for the treatment of non-Hodgkin's lymphoma (B-NHL) [7]. More recently, a fully humanized CD20 antibody, Ofatumumab (OFA, trade name Arzerra), was approved by FDA for the treatment of chronic lymphocytic leukemia (CLL) [8–10]. OFA has also shown potential for treating follicular non-Hodgkin's lymphoma, diffuse large B cell lymphoma and some other CD20-positive B lymphoid malignancies. Generally, the efficacy of OFA can be enhanced by combination with cytotoxic chemotherapy or biologic agents [9, 11, 12], yet OFA directly conjugated with drugs for drug delivery or anti-tumor activity has not been reported.

To understand whether cytotoxic drug conjugation could enhance the therapeutic effect of CD20-targeted OFA antibody, OFA-vcMMAE was synthesized in our laboratory by conjugating OFA with highly cytotoxic monomethyl auristatin E (MMAE) via a valine-citrulline (vc) peptide linker. Our data clearly showed that the initial binding affinity of OFA, complement-dependent cytotoxicity (CDC), and antibody-dependent cell-mediated cytotoxicity (ADCC) were not affected by conjugation of anti-CD20 mAb OFA with MMAE compared with unconjugated mAb OFA. Surprisingly, OFA-vcMMAE significantly enhanced the cytotoxic activity of OFA against CD20-positive B lymphoma cells in vitro, and completely inhibited the growth of Daudi and Ramos lymphoma xenografts in vivo, suggesting that MMAE conjugation may be used as a potential approach to improve the effectiveness of OFA in the treatment of CD20-positive B lymphoid malignancies.

Materials and methods

Reagents

Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories (Shanghai, China). vcMMAE was generously gifted by Concortis Biosystems, Corp. (San Diego, California). Herceptin (IgG, control), Rituxan (anti-CD20 mAb), TGLA (anti-CD20 mAb) and OFA were kindly provided by Zhejiang Hisun Pharmaceutical Co. Ltd.. Annexin V-FITC Apoptosis Detection Kit, BCA protein assay kit, Rabbit anti-cleaved caspase-3 monoclonal antibody, mouse anti-caspase-3 monoclonal antibody, rabbit anti-Poly (ADP-ribose) polymerase (PARP) polyclonal antibody, FITC-labeled goat anti-human IgG (H+L) polyclonal antibody, HRP-labeled goat anti-mouse IgG (H+L) polyclonal antibody, HRP-labeled goat anti-rabbit IgG (H+L) polyclonal antibody and Alexa Fluor 555-labeled goat anti-mouse IgG (H+L) polyclonal antibody were purchased from Beyotime (JiangSu, China). Mouse monoclonal antibody against LAMP-1 and mouse monoclonal antibody against β-actin were purchased from Santa Cruz Biotechnology, Inc. (Delaware Avenue Santa Cruz, USA). All other chemicals and reagents were from Sigma-Aldrich (St. Louis, USA).

Cell culture

CD20-positive Human WIL2-S, Raji, Daudi, Ramos and CD20- negative K562 cells were purchased from American Type Culture Collection, and maintained in RPMI-1640 medium supplemented with 10 % fetal bovine serum at 37 °C in 5 % CO_2 atmosphere.

Preparation of antibody-drug conjugates (ADCs)

OFA-vcMMAE or Herceptin-vcMMAE was synthesized according to published methods [13, 14]. Briefly, 2 mL antibody (10 mg/mL) was mixed with 40 μL of 20 mM Tris (2-chloroethyl) phosphate (TCEP) and then incubated for 2 h at 37 °C. After the incubation, the antibody solution was diluted with PBS to 5 mg/mL and chilled on ice immediately. 133 μL prechilled 10 mM vcMMAE/30 % acetonitrile was added rapidly to the reduced antibody solutions, and the mixture

was left on ice for 1 h. Reactions were quenched by incubating with a 20-fold excess of L-Cysteine over maleimide. In addition, ADC in the reaction mixtures was purified on a pre-equilibrated HiPrep 26/10 Desalting (GE) column. Concentration of ADC was determined by UV absorption at 248 nm and 280 nm, and the drug/Ab ratio was also determined by measuring the unreacted thiols with 5,5'-dithiobis (2-nitro benzoicacid) (DTNB) after re-reduction with TCEP. In addition, aggregation and residual free drug were determined by size-exclusion chromatography and reverse-phase HPLC respectively.

Binding of Anti-CD20 and Anti-CD20 ADC to CD20–positive B Cells

Binding of OFA and OFA-vcMMAE to CD20-positive human B cells was determined by flow cytometry. 1×10^6 cells were incubated with OFA, or with the corresponding conjugate in buffer (containing PBS, 1 % bovine serum albumin (w/v)) for 30 min on ice. After incubation, cells were washed twice with PBS and then incubated with FITC-labeled goat anti-human IgG (H+L) polyclonal antibody at 1:200 for 30 min on ice. After washing, cells were examined on a Beckman Coulter Cytomics FC500 Flow Cytometry. Data analysis was performed using CXP Analysis 2.2 (Beckman Coulter) and the geometric mean fluorescence intensity ratio (MFI) of each cell line was determined.

Assessment of CDC

Cells were seeded at a density of 40,000–60,000 cells/well in 96-well microtiter plates (Corning). After seeding, cells were exposed to various concentrations of OFA or OFA-vcMMAE in the presence or absence of human serum (1:40 final dilution) at 37 °C in a humidified atmosphere of 5 % CO_2 for 2 h. Then, cell viability was determined by Cell Counting Kit-8 [15]. The absorbance of 450 nm was measured by BioRad Model 680 Microplate Reader.

Assessment of ADCC

Briefly, 1×10^4 CD20-positive B cells were incubated with different concentrations of OFA or OFA-vcMMAE for 30 min in phenol-red-free 1640 culture medium, followed by incubating with 2.5×10^5 human peripheral blood mononuclear cells (PBMC) as effector cells at a effector cell to target cell ratio (E:T) of 25:1 for 5 h at 37 °C. The amount of lactate dehydrogenase (LDH) in the cell-free culture medium was assessed using the CytoTox 96 non-Radioactive Cytotoxicity Assay kit (Promega) according to the manufacturer's instructions. Cells were lysed with Triton X-100 to assess the maximum LDH release. The percent cytotoxicity was calculated according to the following formula: % lysis=(experimental release-spontaneous release)/(maximum release – spontaneous release)×100.

Determination of cytotocixity

Cells were seeded at a density of 1×10^4 cells/well in 96-well microtiter plates (Corning). After seeding, cells were exposed to various concentrations of OFA or OFA-vcMMAE at 37 °C for 96 h. Cell viability was determined by Cell Counting Kit-8. The absorbance of 450 nm was measured by BioRad Model 680 Microplate Reader.

Competitive inhibition

Competitive inhibition of the ADC on cells was performed to determine whether the conjugate exhibited cytotoxic activities through ADC-CD20 binding. Cells were supplemented with 1 μg/ml of OFA-vcMMAE in the presence of 100 μg/ml of OFA at 37 °C for 72 h. The cell viability was assessed using CCK-8 kit as mentioned above.

Analysis of internalization

Internalization of cell surface-bound mAb or its conjugate was assessed by a method reported elsewhere [16]. WIL2-S cells were saturated with excess OFA or OFA-vcMMAE (10 μg/mL) in culture medium at 4 °C for 30 min. After washing, cells were incubated at either 4 °C (with NaN_3) or 37 °C for 2 h to drive internalization. The internalization reaction was stopped by washing with cold PBS, and then cells were determined for mAbs internalization or CD20 internalization.

mAbs internalization

Cell surface-bound OFA or OFA-vcMMAE were stained with FITC-labeled goat anti-human IgG (H+L) polyclonal antibody at 4 °C for 30 min and the density of binding was then examined by flow cytometry as described above. The percentage of internalization of antibody or ADC was determined by using the MFI and the following formula: % internalized=total surface bound (4 °C)–total surface bound (37 °C)/total surface bound (4 °C)×100 .

CD20 internalization

Cells were reconstituted for 5 min in RPMI 1640 medium containing 10 % fetal bovine serum at pH 2.5, a condition that promoted release of OFA or OFA-vcMMAE that was bound to the cell surface [17]. After acid washing for three times, cells were incubated with OFA (10 μg/mL) and then FITC-labeled goat anti-human IgG (H+L) polyclonal antibody on ice for 30 min as an alternative measure of CD20 on the cell surface

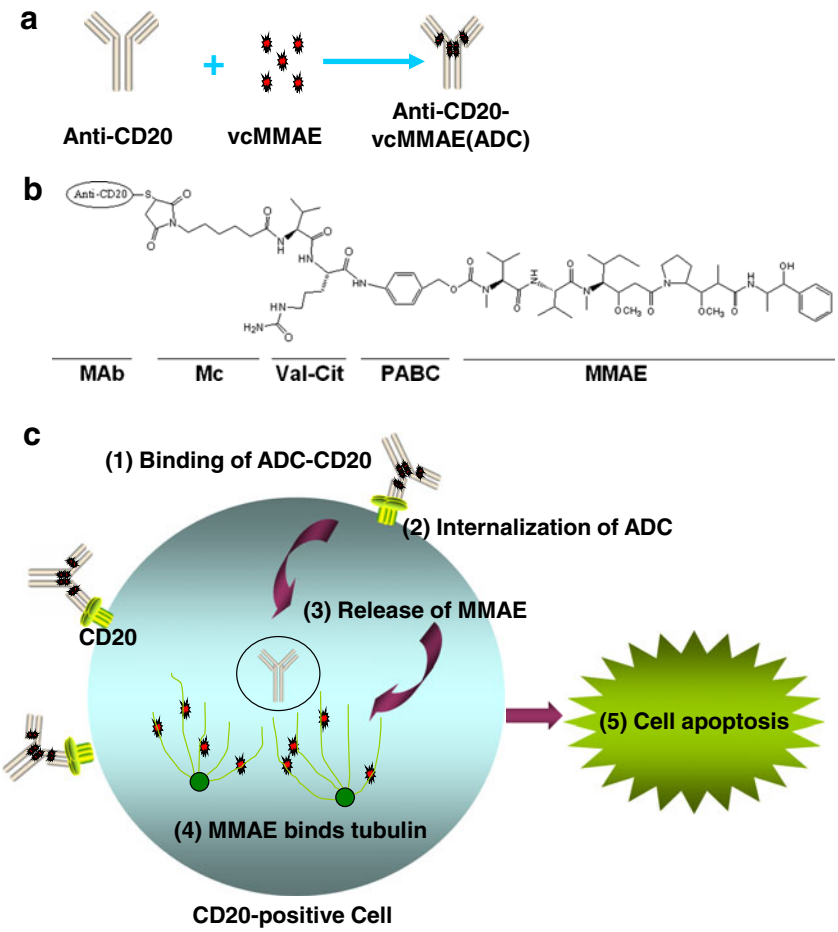


Fig. 1 OFA-vcMMAE delivering MMAE to CD20-positive cancer cells. **a** Process of OFA-vcMMAE conjugation synthesis steps. **b** The chemical structure of OFA-vcMMAE. **c** Proposed mechanism for anti-cancer effect of OFA-vcMMAE on CD20-positive cancer Cells

by flow cytometry as described above. The percentages of internalized CD20 antigen were calculated based on the following formula: % internalized=total surface bound (untreated control cells)–total surface bound (mAb or ADC treated cells)/total surface bound (untreated control cells)×100.

Microscopy for ADC trafficking

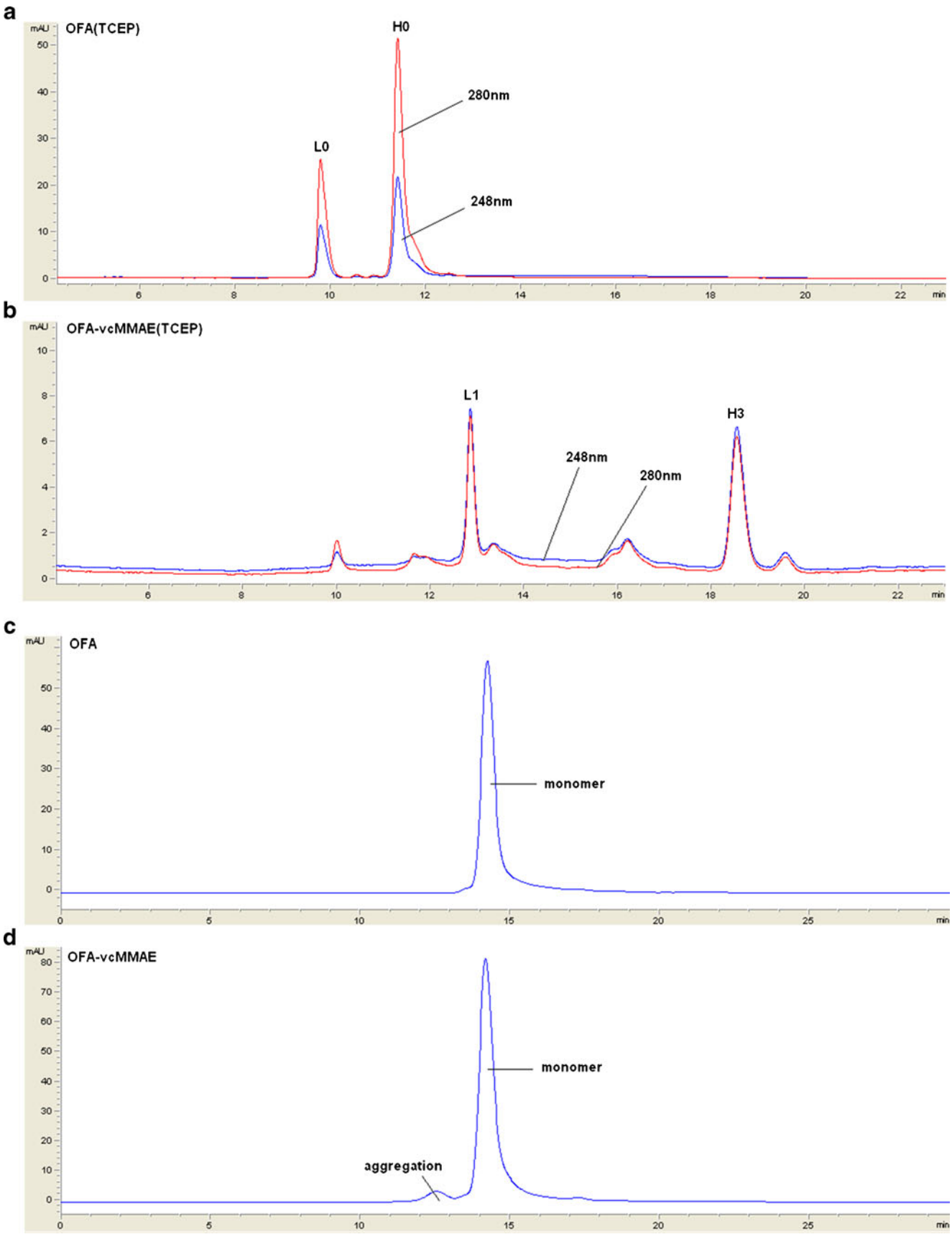
WIL2-S cells seeded at 5×10^5 cells/ml were treated with 5 μg/ml of OFA-vcMMAE at 37 °C for 4 h. OFA-vcMMAE was detected with FITC-labeled goat anti-human IgG (H+L) polyclonal antibody, lysosomes with mouse monoclonal antibody against LAMP-1 followed by Alexa Fluor 555-labeled goat anti-mouse IgG (H+L) polyclonal antibody, nuclei with 4',6-diamidino-2-phenylindole (DAPI). Cells were examined using a Zeiss LSM 510 Meta Confocal Microscope. Images were captured with a digital camera and LSM510 software.

Determination of apoptosis and immunoblot analysis

WIL2-S cells were seeded at a density of 5×10^4 cells/ml and then exposure to OFA, Rituxan, TGLA [18] or OFA-vcMMAE at a concentration of 5 μg/ml for 72 h. The control group was incubated with medium alone. After exposure, cells were collected and stained with AnnexinV-FITC and PI for apoptosis analysis. The percentages of apoptotic cells (AnnexinV+/PI-) and dead cells (AnnexinV+/PI+) were determined by flow cytometric analysis of each population [19].

Regarding the immunoblot analysis, drug–exposed cells were re-suspended in RIPA buffer (containing Tris 50 mM, Triton X-100 1 %, SDS 0.1 %, NaCl 150 mM, EDTA 1 mM, sodium deoxycholate 1 %, aprotinin 1 μg/ml, PMSF 1 mM and leupeptin 0.5 μg/ml, pH7.4) and centrifuged at 14,000*g* at 4 °C for 15 min. The protein concentrations in the supernatant were determined using the BCA protein assay kit. Proteins were resolved by 10 or 12 % SDS-PAGE and electroblotted onto nitrocellulose membranes (Bio-Rad, Mississauga, ON). The membranes were blocked for 1 h at room temperature in PBS containing 5 % skim milk plus 0.1 % Tween-20 and incubated overnight at 4 °C with rabbit

Fig. 2 Typical chromatograms of OFA-MMAE for the synthesis process. Reduction of OFA by TCEP (**a**) or conjugation of reduced OFA with vcMMAE (**b**) was determine and monitored by PLRP-S reversed-phase columns at wavelengths of 248 nm (λ_{max} for MMAE) and 280 nm (λ_{max} for Ab). Peak L0 and H0 indicate the light chain and heavy chain (**a**), and L1 and H3 represent the light chain and heavy chain conjugated with MMAE (**b**). In addition, OFA (**c**) and OFA-vcMMAE (**d**) were further determined by size-exclusion chromatography for evaluation of aggregation


a
OFA(TCEP)
H0
280nm
L0
248nm
b
OFA-vcMMAE(TCEP)
L1
H3
248nm
280nm
c
OFA
monomer
d
OFA-vcMMAE
monomer
aggregation

anti-cleaved caspase-3 monoclonal antibody, mouse anti-caspase-3 monoclonal antibody, rabbit anti-PARP polyclonal antibody or mouse monoclonal antibody against β-actin followed incubation with HRP-labeled goat anti-mouse IgG (H+L) polyclonal antibody or HRP-labeled goat anti-rabbit IgG (H+L) polyclonal antibody. Protein bands were visualized with EZ-ECL Kit (Biological Industries, Israel).

Animals experiment

All animal experiments were carried out according to the "Principles of Laboratory Animal Care" (NIH version, revised 1996) and the Guidelines of the Animal Investigation Committee, College of Pharmaceutical Sciences, Zhejiang University, China (Permit No: ZJU2010101033). 5 week old male C.B.-17 SCID mice and 4–5 week old male nude mice were purchased from National Rodent Laboratory Animal, Resource, Shanghai, China. They were housed in a humidity-controlled room, maintained at 22–25 °C with a 12 h light–dark cycle. The animals were fed a commercial diet and tap water ad libitum. Following a 1-week acclimatization period, mice were used for experiments.

Antitumor activities of OFA-vcMMAE in in vivo

Tumor implantations were performed in 6-week old C.B.-17 SCID mice or 5–6 week old nude mice by subcutaneous injection of 1×10^7 Daudi or Ramos cells (right flanks). Treatment was started in each group when tumors reached mean group size of 260 or 280 mm^3 for SCID mice or nude mice. Each antibody OFA, Herceptin-vcMMAE and OFA-vcMMAE was injected intravenously at the dose of 3 mg ADCs/kg body weight into the two types of mice every 3 days for three times. Free MMAE was at molar equivalent of MMAE present in 3 mg/kg OFA-vcMMAE. Each control group, under identical conditions, was injected the same volume of PBS alone. Tumor sizes were measured with digital calipers, and tumor volumes were calculated by using the formula: $(L\times W^2)/2$, where L is the longest diameter of the tumor and W is the shortest diameter perpendicular to L. Tumors were excised from the euthanized animals in each treatment group (i.e., treatment of PBS, OFA, Herceptin-vcMMAE and MMAE), while the OFA-vcMMAE treated group was left for further study.

Statistical analysis

Each viability value was calculated using GraphPad Prism 5.0 demo (GraphPad Software Inc., San Diego CA). Differences between the experimental groups were tested for significances on the basis of unpaired, one-tail t tests using GraphPad Prism software. A probability value of less than 0.001, 0.01 and 0.05 ($*p<0.05$, $**p<0.01$ and $***$ $p<0.001$) was accepted as a significant difference.

Results

Anti-CD20 ADCs preparation and characterization

OFA-vcMMAE was synthesized in our laboratory by reducing the internal mAbs disulfides with TCEP followed by addition of vcMMAE, as schematically represented in Fig. 1a and b. Moreover, drug (MMAE)/Ab (OFA) ratio for the ADC was calculated to be 7.5, and the resulting conjugate used in these studies exceeded 98 % monomeric protein. The

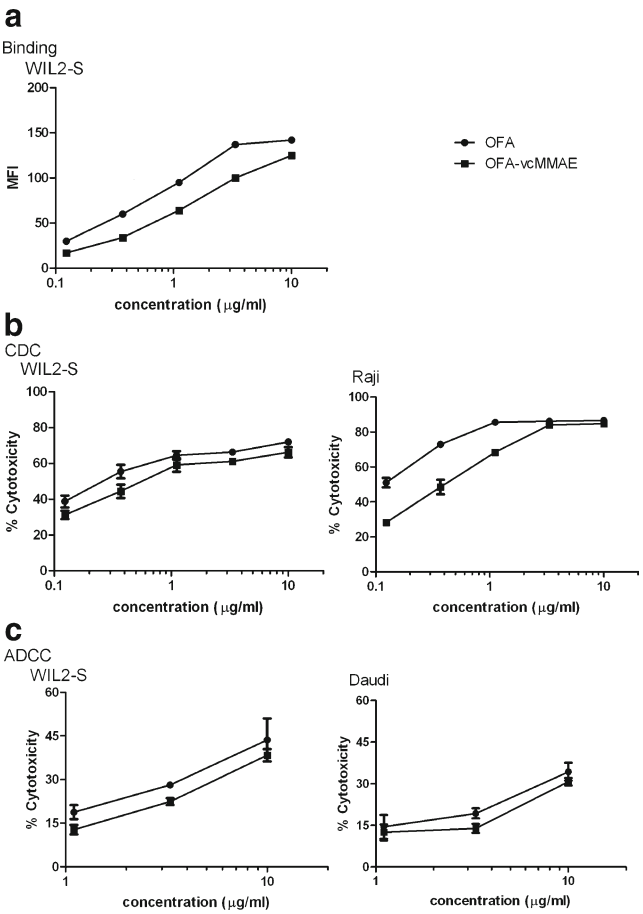


Fig. 3 Determining the binding, CDC and ADCC of mAb and ADC to WIL2-S cells. **a** Cells were exposed to increasing concentrations of mAb or ADC for 30 min. The bound mAb or ADC was stained with FITC-labeled goat anti-human IgG (H+L) polyclonal antibody, and analyzed by flow cytometry (MFI) $n=2$. **b** CD20-positive cells were incubated with various concentrations of mAb or ADC in the presence of human serum (1:40 dilution) as a source of complement. The cell viability was assessed using CCK-8 kit. **c** CD20-positive cells were incubated with various concentrations of mAb or ADC in the presence of mononuclear cells from human plasma as effector cells (effector to target ratio=25). Five hours later, the released LDH activity was determined as an indication of cell lysis. The results are represented as mean ± S.D. from triplicate determinations, one of three similar experiments

Table 1 Comparison of toxic effects of OFA-vcMMAE and unconjugated mAb on CD20-positive and negative cell lines

Cell line	OFA IC_{50}[a, b]	OFA-vcMMAE IC_{50}[a]	Herceptin -vcMMAE IC_{50}[a]	Free MMAE IC_{50}[c]	OFA-vcMMAE IC_{50}[c]
Daudi($CD20^+$)	>667	0.16±0.03	>313	0.62±0.03	1.2±0.2
WIL2S($CD20^+$)	>667	0.32±0.01	>313	0.40±0.07	2.4±0.1
Raji ($CD20^+$)	>667	0.18±0.06	>313	0.41±0.09	1.3±0.5
K562 ($CD20^-$)		>31	–	0.50±0.23	>230

[a] IC_{50} values are expressed as nmol/L. All Data are expressed as the mean ± S.D. from three independent experiments performed in triplicate

[b] Doses are expressed as equivalents of antibody

[c] Doses are expressed as equivalents of MMAE

level of free drug in the ADC was below 0.5 % (below limit of quantitation)..

The typical chromatograms of OFA-vcMMAE for the synthetic process by PLRP-S reverse-phase column were shown in Fig. 2a–d. Antibody (i.e., unconjugated) was reduced by TCEP (Fig. 2a) and then added vcMMAE to form the OFA-vcMMAE (Fig. 2b). Two major peaks, namely, a light chain (L0) and heavy chain (H0) of OFA were clearly determined after the reduction, and the two major peaks were shifted to right side by addition of vcMMAE, suggesting the two chains conjugated (i.e., L1 and H3) with MMAE (Fig. 2b). In addition, one MMAE in L1 chain and three MMAE in H3 chain were also observed according to the ratio of absorbance at wavelengths of 248 nm and 280 nm[20].

On the other hand, size-exclusion chromatography was used to determine the formation of aggregation after synthesis. Figure 2c and d shows the chromatograms of the mAb before (c) and after the conjugation with MMAE (d), and no obvious aggregation was observed after conjugation with MMAE (less than 2 %), suggesting the conjugated mAb remains the original structure. Regarding the binding affinity, conjugation of OFA with MMAE caused a modest decrease in the binding affinity to cell surface CD20 (Fig. 3a), while synthesized OFA-vcMMAE did not bind to CD20-negative K562 cells (data not shown), suggesting that OFA-vcMMAE could specially bind to CD20 positive cells. The negative control, Herceptin-vcMMAE (Drug/Ab ratio 7.3), also did not bind to CD20-positive cells (data not shown).

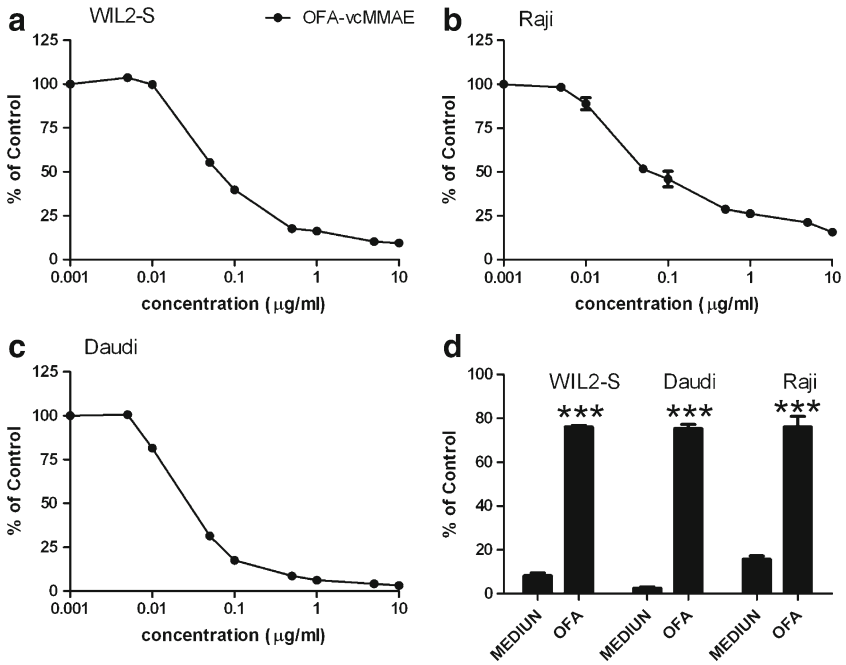


Fig. 4 OFA-vcMMAE inducing a potent antiproliferative effect in CD20-positive tumor cells. WIL2-S (**a**), Raji (**b**), and Daudi (**c**) were all treated with ADC for 96 h, and relative proliferation rates were determined using CCK kit. Cell viability was assessed by detecting fluorescence in 96-well plates and compared with that of complete medium alone (control). Data points represent mean ± S.D. for triplicate determinations, one of three similar experiments. **d** To determine whether ADC exhibited activity through ADC-CD20 binding, WIL2-S, Raji and Daudi cells were exposed to 1 μg/ml OFA-vcMMAE in the presence of 100 μg/ml OFA for 72 h. The cell viability was assessed using CCK-8 kit as described in Materials and methods. The results are represented as mean ± S.D. from triplicate determinations, one of three similar experiments. *Asterisks* (*) indicate significant difference from the control (incubation with ADC alone) at ***p <0.001

The impact of the conjugation of OFA with MMAE on its ability to mediate ADCC and CDC was also examined in the present study. Conjugation of OFA to MMAE caused a slight decrease in its ability to mediate CDC (Fig. 3b) and ADCC (Fig. 3c). In spite of this, OFA-vcMMAE still exhibited CDC and ADCC activities against CD20-positive B cells.

Determination of cytotoxicity in different cell lines

To evaluate the cytotoxicity of OFA-vcMMAE, the CD20-positive and negative cell lines were exposed to OFA, OFA-vcMMAE, MMAE and Herceptin-vcMMAE for 96 h. The results showed that OFA was lowly cytotoxic to three CD20-positive cells, and the IC_{50} values were calculated to be higher than 667 nmol/L (Table 1), and free MMAE exhibited high cytotoxicity against both CD20-positive and negative cell lines (Table 1). On the other hand, OFA-vcMMAE showed only high toxicity to CD20-positive cancer cells, while there is no appreciable toxic effect observed on the CD20-negative K562 cells, suggesting the cytotoxicity of OFA-vcMMAE is specific for CD20-positive cells (Table 1 and Fig. 4). Herceptin-vcMMAE was used as a negative control in current experiment.

In addition, the specificity of OFA-vcMMAE mediated growth inhibition was further characterized by a competition experiment, as shown in Fig. 4d. It has been shown that excess unconjugated OFA (100 μg/ml) was able to reduce the growth-inhibitory effects of OFA-vcMMAE (1 μg/ml) in CD20-positive WIL2-S, Daudi and Raji cells. These observations indicate that the unconjugated anti-CD20 Ab can inhibit the binding of OFA-vcMMAE to CD20 antigen and consequently inhibit the cytotoxicity of OFA-vcMMAE.

Determination of internalization and apoptosis in WIL2-S cells after exposure to OFA-vcMMAE

Figure 5a shows the internalization rates of OFA or the corresponding anti-CD20 ADC in the CD20-positive WIL2-S cells. Change in the surface levels of mAb or ADC on cells was determined by flow cytometry after incubation for 2 h at 37 °C. OFA exhibited modest internalization into the WIL2-S cells, which was significantly increased after conjugation with MMAE (Fig. 5a), suggesting that the conjugation of vcMMAE could facilitate the internalization of OFA in WIL2-S cells. To ensure that the conjugate is internalized into cells in the form of OFA-vcMMAE/CD20 complexes, we used an alternative determination of CD20 on the cell surface after partial internalization of bound mAbs. Figure 5b shows the treatment with OFA-vcMMAE for 2 h, which resulted in a reduction of more than 20 % of CD20 on the surface of WIL2-S cells, while treatment with unconjugated mAb left CD20 unchanged (Fig. 5b). Moreover, the amount of internalized OFA-vcMMAE (Fig. 5a) was very close to that of internalized CD20 antigen (Fig. 5b). The

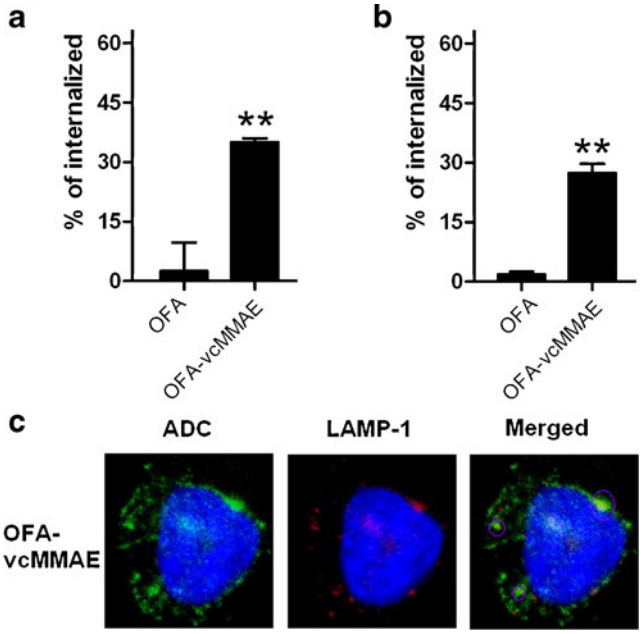


Fig. 5 OFA-vcMMAE internalizing and trafficing to the lysosomes in CD20-positive WIL2-S cells. CD20-positive WIL2-S cells were exposed to OFA or OFA-vcMMAE conjugates for 30 min at 4 °C. Unbound mAb or ADC was removed by washing with PBS. Cells were incubated at 37 °C or 4 °C with NaN_3 for 2 h. Cell surface-bound mAb or ADC was detected with FITC-labeled goat anti-human IgG (H+L) polyclonal antibody by flow cytometry. The percentage of internalized mAb or ADC was calculated as described in the Materials and methods (**a**, n=6). MAb or ADC exposed-cells were washed with acid buffer for determination of changes in CD20 antigen on cell surface. Cells were incubated with OFA (10 μg/ml) and then FITC-labeled goat anti-human IgG (H+L) polyclonal antibody on ice for 30 min to measure the loss of surface CD20 in vitro as described above (**b**, n=4). The p value is indicated as follows: $^{*}p<0.05$, $^{**}p<0.01$, or $^{***}p<0.001$, compared with corresponding mAb group. **c** WIL2-S cells were treated with ADC in complete medium at 37 °C for 4 h. Cells were fixed, permeabilized and cell-associated ADC and lysosome-associated membrane protein LAMP-1 was detected with respective fluorochrome-conjugated secondary Abs. Nuclei were stained with DAPI. ADC (*green*) with lysosome (*red*) on WIL2-S cells were co-localized (*yellow*) and indicated by circles

internalization of OFA-vcMMAE in cells was further determined using confocal microscope as shown in Fig. 5c. The ADC was localized inside the cells after 4 h exposure, verifying its internalization. In addition, the ADC was also co-localized with lysosomal-associated membrane protein 1 (Lamp-1), suggesting that OFA-vcMMAE reached the lysosomal compartment.

Induction of apoptosis in WIL2-S cells was determined by Annexin V/PI staining with flow cytometry following exposure to OFA-vcMMAE at a concentration of 5 μg/ml for 72 h, as shown in Fig. 6a. The percentages of apoptotic cells and dead cells (i.e., late apoptotic cells) in OFA-vcMMAE-treated cells reached to 23.5 % and 38.4 % respectively, while unconjugated OFA-treated cell showed low percentages of apoptosis, suggesting that synthesized ADC achieved in specific delivery of MMAE to CD20 positive cells (Fig. 6a). In addition, the

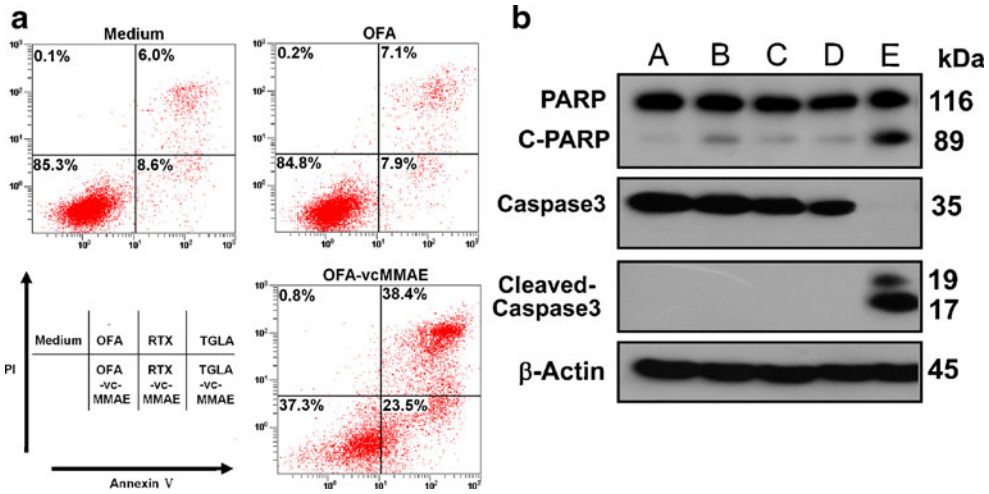


Fig. 6 Effect of OFA-vcMMAE on induction of apoptosis in WIL2-S cells. **a** WIL2-S cells were exposed to Ab, or ADC at 5 μg/mL for 72 h. The control group was incubated with medium alone. Induction of cell apoptosis was detected using Annexin V-FITC (FL1 channel) and PI-Staining (FL2 channel) by flow cytometry. Damaged cells: Annexin V-FITC negative/PI positive (*upper left*). Late apoptotic and necrotic cells: Annexin V-FITC positive/PI positive (*upper-right*). Live cells: Annexin V-FITC negative/PI negative (*lower left*). Early apoptotic cells: Annexin V-FITC positive/PI negative (*lower right*). Data are representative of two separate experiments. **b** WIL2-S cells were exposed to Ab, or ADC at 5 μg/mL for 72 h. Changes in the apoptosis-related proteins caspase-3 and PARP were detected by western blot. Proteins (25 μg) extracted from whole cell were separated by electrophoresis on a 10 % or 12 % SDS-polyacrylamide gel as described in Materials and methods. Line A, Medium; B, OFA; C, Rituxan; D, TGLA; E, OFA-vcMMAE. Actin was used as a loading control

apoptosis related proteins such as caspase-3, cleaved caspase-3, Poly (ADP-ribose) polymerase (PARP), and cleaved PARP, were also determined (Fig. 6b). Cleaved caspase-3 and PARP were clearly detected in WIL2-S cells after exposure to OFA-vcMMAE for 72 h (Fig. 6b, Lane E) as compared with medium alone (Fig. 6b, Lane A) and three unconjugated anti-CD20 antibodies, OFA, Rituxan and TGLA-treated groups (Fig. 6b, Lane B, C, D).

Based on the above results, binding of OFA-vcMMAE to WIL2-S might possibly accelerate the modulation of CD20 antigen, resulting in the internalization of OFA-vcMMAE/CD20 complexes into the lysosomes. Then, internalized OFA-vcMMAE released free MMAE and induced apoptotic cell death through a caspase-3-like protease-dependent pathway.

Determination of Anti-tumor Activity of OFA, OFA-vcMMAE, MMAE and Herceptin-vcMMAE in vivo

In order to evaluate the therapeutic potential of OFA-vcMMAE in vivo, the anti-tumor activity of ADC was determined in Daudi human lymphoma xenografts model and Ramos human lymphoma xenografts model.

When tumor volumes (TV) reached approximately 260 mm^3, 18 SCID mice were randomly divided into five treatment groups. As results, untreated control group (n=3) and mice treated with MMAE (n=3), OFA (n=3) and Herceptin-vcMMAE (n=3) developed progressive tumor growth with an average TV of more than 1,200 mm^3 within 8 days after injection (Fig. 7), suggesting OFA, MMAE and Herceptin-vcMMAE have no inhibitory effect on tumor growth. Surprisingly, the tumor has completely disappeared in all mice at day 8 in OFA-vcMMAE-treated group (n=6), indicating the conjugation of vcMMAE with OFA is able to improve its anti-cancer effects against CD20-positive B lymphomas. In addition, the tumor growth in OFA-vcMMAE-treated mice was also determined during day 8–27. As expectedly, no tumor recurrence was observed in all six mice.

Similar results were also observed in the nude mice bearing Ramos human lymphoma xenografts, as shown in Fig. 8. The tumors (TV ≥280 mm^3) completely vanished after injection of OFA-vcMMAE at day 8 in all six mice, and no tumor recurrence were observed in all six nude mice at 40-days. On the other hand, OFA (n=3) alone had little inhibition effect on Ramos human lymphoma xenografts nude mice, while MMAE and Herceptin-vcMMAE (n=3) did not inhibit the tumor growth (tumor volumes≥1,595 mm^3), which was similar to PBS treated group at 8 days after injection.

Discussion

Antibody-drug conjugates (ADCs) are emerging as a powerful class of anti-tumor agents with efficacy across a range of cancers. Especially, anti-CD20-based ADCs are recently considered to be the promising anticancer agents if conjugated to highly potent cytotoxic drugs [16]. However, one premise of this drug delivery technology is that ADCs should be internalized, traffic to the lysosomes, and metabolized by lysosomal proteases to release free drugs [21,22].

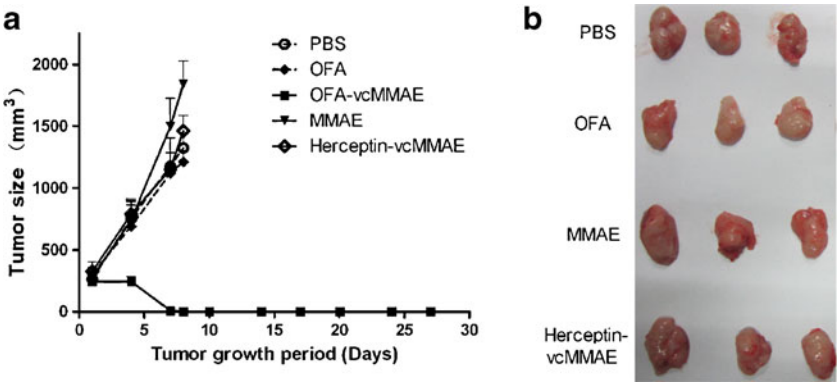


Fig. 7 Determination of anti-tumor activities of OFA, OFA-vcMMAE, MMAE and Herceptin-vcMMAE in Daudi xenografts. SCID mice bearing Daudi lymphoma cells xenograft were intravenously injected with OFA (n=3), OFA-vcMMAE (n=6) and Herceptin-vcMMAE (n=3) at dose of 3.0 mg Ab or ADCs/kg body weight, and MMAE (n=3) at 0.11 mg MMAE/kg body weight via the tail vein every three day for 3 times, as described in material and method. Treatment was started when tumor volumes reaching approximately 260 mm^3. Control group was injected the same volume of PBS. **a** The tumor volumes were calculated by measurements of length and width from each treatment group. **b** Subcutaneous tumors in SCID mice were excised at day 8

In fact, anti-CD20 mAbs conjugated with certain cytotoxic drugs or toxins (e.g., doxorubicin or ricin A-chain) against CD20-positive B lymphoma cells has been evaluated in previous studies. However, these ADCs were found to be ineffective, and this ineffectiveness was attributed to the difficulty of internalization of anti-CD20 antibody (Ab)/CD20 complexes into the CD20-positive B lymphoma cells [23, 24]. Recent reports have shown that, although unconjugated anti-CD20 antibody Rituximab was hard to be internalized into CD20-positive cells, the internalization ability and anti-tumor activity of Rituximab can be significantly enhanced by conjugating with vcMMAE [16]. Ofatumumab (OFA) is the first fully human anti-CD20 mAb approved by U.S. FDA for patients with chronic lymphocytic leukemia (CLL). It has shown promising activity against a broad range of CD20-positive B-cell malignancies [9, 25]. Regarding the antigen-binding sites for different CD20 antibodies, it has been found that Ofatumumab is able to bind to a novel epitope, namely, both the small and large loops of CD20, while most others (e.g., Rituximab) only bind to large loop of CD20 [26]. In addition, researchers have developed a few combination chemotherapy regimens to improve the anti-cancer activity of OFA using chemotherapeutic agents such as fludarabine, lenalidomide, cyclophosphamide or chlorambucil, but the anticancer effects were not always enhanced satisfactorily [11].

In the current study, in order to increase the anti-cancer effect of OFA, we determined whether the cytotoxicity of OFA could be increased by conjugating with vcMMAE. As expectedly, the cytotocixity of OFA was improved by conjugation with MMAE in CD20-positive malignant B cells compared with unconjugated OFA (Fig. 4a, b and c). In addition, cytotoxicity of OFA-vcMMAE can be completely reduced in the presence of excess anti-CD20 antibody OFA (Fig. 4d), suggesting that the cytotoxicity of OFA-vcMMAE predominantly exerts in intracellular after binding to CD20. Regarding the internalization, we found that OFA is initially

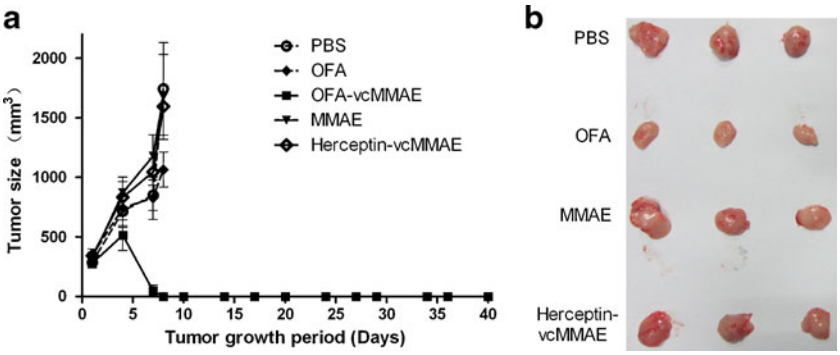


Fig. 8 Determination of anti-tumor activities of OFA, OFA-vcMMAE, MMAE and Herceptin-vcMMAE in Ramos xenografts. Nude mice bearing CD20-positive Ramos lymphoma cells xenograft were intravenously injected with OFA (n=3), OFA-vcMMAE (n=6) and Herceptin-vcMMAE (n=3) at dose of 3.0 mg Ab or ADCs/kg body weight, and MMAE (n=3) at 0.11 mg MMAE/kg body weight via the tail vein every three day for 3 times, as described in material and method. Treatment was started when tumor volumes reaching approximately 280 mm^3. Control group was injected the same volume of PBS. **a** The tumor volumes were calculated by measurements of length and width from each treatment group. **b** Subcutaneous tumors in nude mice were excised at day 8

difficult to be internalized into WIL2-S cells, but the internalization capability can be markedly improved by conjugation with MMAE (Fig. 5a). Our result is also consistent with Law's study that conjugation with vcMMAE facilitates its internalization ability of Rituximab [16]. CD20 internalization with anti-CD20 mAb ligation has been demonstrated previously [27]. On the other hand, CD20 modulation of WIL2-S was observed after treatment with OFA-vcMMAE rather than OFA (Fig. 5b). Therefore, improved internalization of anti-CD20-vcMMAE might depend on the receptor-mediated endocytosis, but not on other non-specific endocytosis which might happen with the modified Ab.

Indeed, trafficking of OFA-vcMMAE to the lysosomal compartment could undergo proteolytic degradation and release active drug MMAE to kill CD20-positive lymphoma cells (Figs. 5c and 6). It has been reported that the MMAE-related dolastatin 10 and auristatin PE induce great growth arrest and apoptosis [16, 28]. Similar results were also obtained in WIL2-S cells after exposure to OFA-vcMMAE. Apoptosis was significantly induced by OFA after conjugation with MMAE, and no appreciable effect was observed in unconjugated OFA-treated cells (Fig. 6a). Moreover, it has been found that the cleaved caspase-3 and PARP were clearly detected after exposure to OFA-vcMMAE, but not by three unconjugated anti-CD20 antibodies OFA, Rituxan and TGLA, suggesting that conjugation of MMAE with anti-CD20 mAb could increase the rate of apoptosis through a caspase-3-like protease-dependent pathway (Fig. 6b).

In vivo experiments have shown that OFA-vcMMAE, a CD20-targeting anti-cancer drug conjugate, completely inhibited the growth of Daudi and Ramos lymphoma tumor xenograft in mice (Figs. 7 and 8), while OFA, MMAE and Herceptin-vcMMAE (negative control) did not inhibits the tumors growth, suggesting that OFA-vcMMAE is able to deliver MMAE into CD20-positive tumor tissue and improve the anti-tumor activity of its anti-CD20 antibodies against CD20-positive B lymphoid malignancies.

Conclusions

In summary, cytotoxicity of OFA which selectively targets the CD20-positive cancer cells was significantly improved by conjugation with a highly potent cytotoxic agent MMAE which can inhibit cell division by inhibiting tubulin polymerization in vitro. Likewise, OFA-vcMMAE exhibited much stronger anti-tumor activity to CD20-positive lymphoma tumor xenograft in mice in vivo than unconjugated OFA, indicating that conjugation of high-toxic drugs have the potential to become a promising improvement strategy to increase the anti-cancer activity of Ofatumumab for CD20-positive B lymphoid malignancies treatment.

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Competing interests The authors declare that there are no conflicts of interest.

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