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Recombinant cucurmosin-based immunotoxin targeting HER-2 with potent in vitro anti-cancer cytotoxicity

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ABSTRACT

Trastuzumab is a humanized monoclonal antibody against HER2 approved by FDA for breast and gastric cancer therapy. However, only a quarter of patients have the potential to benefit from it, and most of them develop resistance within therapy. The main purpose of this study is to broaden trastuzumab's therapeutic window by conjugating trastuzumab with recombinant cucurmosin to form an immunotoxin called T-CUS_{245C}. T-CUS_{245C} was chemically conjugated and the purification of T-CUS_{245C} was evaluated by SDS-PAGE. SRB tests showed a remarkable cytotoxicity of T-CUS_{245C} with IC_{50} values in picomolar range on HER2 positive cancer cells without significantly proliferation inhibition on HER2 negative cells ($P < 0.01$). Confocal microscopy verified the time-dependent internalization effects of T-CUS_{245C} and revealed that the lethal efficacy can be increased by provoking the internalization. These results indicate the therapeutic potential of T-CUS_{245C} for the HER-2 targeted therapy.

1. Introduction

Immunotoxins (ITs) are antibody-cytotoxin compositions that have been under development for the treatment of cancers for several decades, and hundreds of studies have exhibited the potential application of ITs in pre-clinical studies and in clinical trials [1,2]. Typically, the ITs are comprised of an antibody or an antibody fragment, a cytotoxic drug like a bacterial protein or a plant-derived ribosomal inactivating protein (RIP), and a suitable linker [3]. With the targeting characteristics of antibodies and cytotoxicity of payloads, immunotoxins are supposed to be effective in treating cancers. However, very few of them have reached to the clinic. The main issue impeding the clinical utility of immunotoxins is the off-target side-effects caused by an unstable linker [2]. When toxins are released from the delivery vehicles before getting to target cells, they tend to bind to normal tissues, leading to unexpected cellular necrosis [4]. Besides, the heterogenicity of ITs as a foreign substance is likely to cause systemic auto-immune diseases, like severe dermatitis and has a potential to generate neutral antibodies result in cancer relapse [5–7]. Therefore, to achieve a higher anti-tumor response, a potent toxic payload and an intact antibody against tumor associated antigen (TAA) are not enough. Understand how they function as cell killing agents and how to promote their anti-tumor efficacy is of great priority.

HER2 is a member of ErbB family that has been considered as a critical regulator in cell proliferation and survival. Its mutation or amplification is involved in enhanced aggressiveness of multiple kinds of malignancies [8]. Trastuzumab is a humanized monoclonal antibody against HER2/neu protein. It has been approved by FDA in the therapy of treating breast cancer and gastric cancer [9,10]. Unfortunately, only a quarter of patients have the possibility to benefit from anti- HER2 treatments, and a large part of them

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become resistant to this target therapy within a short period of time [11]. Although, the efficacy of trastuzumab is limited, it is a promising delivery candidate for ITs and it can exert its function by killing cells directly instead of signaling inhibition through being joined to a toxin [1].

Cucurmosin (CUS) is a typical type I RIP plant-derived cytotoxin without unspecific cellular adherence caused by lectin properties [12]. It was isolated by our group from Cucurbita moschata (pumpkin) which function through inhibition of protein synthesis based on a unique catalytic mechanism [13–15]. Our previous study had successfully determined its DNA sequence and amino acid sequence, as well as the tertiary structure [16]. We also found out that CUS is capable of inhibiting the proliferation of various tumor cells significantly in vitro with cytotoxicity 4 to 7 times stronger than that of other RIPs, such as luffaculin and trichosanthin.

In this study, we conjugated trastuzumab to modified CUS_{245C} (a recombinant CUS which a cysteine residue was inserted onto 245th amino acid sequence) by chemical linking and evaluated its anti-tumor activity in multiple cell lines with different HER2 expression level. Then we characterized its biological activity applying SDS-PAGE, Western blotting and flow cytometry, and assessing their cytotoxicity against variety of cancer cell lines, and then investigated the correlation of biological characteristics in terms of internalization utilizing confocal microscopy. The promising results showed an almost fully retained antitumor efficacy in the picomolar range against antigen positive cancer cells. Simultaneously, using confocal microscopy we verified that the lethal effect is mediated by antibody internalization and cytotoxin intracellular degradation. This study provided a novel IT targeting HER2 using self-produced type I RIP CUS and remarkable improved antitumor effects of recombinant IT which represents a potentially attractive therapeutic modality for cancer treatment.

2. Materials and methods

2.1. Cell lines and reagents

2.1.1. Cell line

Human breast cancer cell lines MDA-MB-231, MCF-7, SK-BR-3, BT-474, and human ovarian carcinoma cell lines SKOV-3 were obtained from ATCC. All cells were cultured in DMEM (Gibco, USA) supplemented with 10% FBS (Gibco, USA).

2.1.2. Reagents

pET-32a vector (+) and *E.coli* BL21 (DE3), his-tagged fusion protein kit (Sangon Biotech, Shanghai). Trastuzumab (Roche). Mouse anti-human IgG1-APC, mouse anti-his-FITC were purchased from BD Bioscience (San Jose, CA). Goat anti-mouse IgG-HRP (Santa Cruz Biotechnology).

2.2. CUS_{245C} expression

We inserted a cysteine residue to the 245th nucleotide sequence of CUS and labeled it with a His-tag. *Escherichia coli* BL21 (DE3) and expression vector pET32a (+) were applied for the expression of the CUS_{245C} according to the manufacturer's protocol. Bacterial cells transferred with the pET32a (+)-CUS_{245C} were cultivated at 37 °C in LB medium at 200 rpm on a rotary shaker until OD_{600} reached 0.8. IPTG (1 mM) were used for expression induction at 25 °C for 16–18 h. Cells were later sonicated (Sonics 5s, pulse10s, Ampl 60%) in PBS containing 0.068 mM Lysozyme for 60min. The lysate was centrifuged twice for 20 min at 4 °C and purified by Ni-NTA. The concentration and molecular masses of the CUS_{245C} were determined by BCA Protein Assay Kit (Beyotime, China) and SDS-PAGE, respectively.

2.3. Conjugation of T-CUS $_{245C}$

10 mg Trastuzumab were dialyze against 20 mM PBS (pH7.5) and mixed with five-fold SPDP (20 mM in DMSO) and incubated at room temperature for 30min. CUS_{245C} dimers were reduced into monomers by 0.5 M Dithiothreitol (DTT, Beyotime) for 30min to expose sulfydryl groups thoroughly. The excess SPDP and DTT were later removed by overnight dialysis. The mixtures were allowed be reacted with four-fold of DTT-reduced CUS_{245C} at 23 °C for 18 h. The conjugating reaction was terminated by 0.1 M iodoacetamide. After centrifuge and dialysis, Float-A-Lyzer G2 with the MWCO of 100 kDa (Spectrum Laboratories, Inc) was utilized to get rid of the CUS_{245C}, while T-CUS $_{245C}$ were collected and purified by 1.0 × 12 cm a Ni-NTA column (packed with 1.2 ml Ni^{+}-NTA resin).

2.4. SDS-PAGE and western blotting

Purified protein was analyzed through 6% SDS-PAGE and 8% SDS-PAGE gel and Western blot analysis used mouse *anti*-CUS antibody as primary antibody and goat anti-mouse IgG-HRP as the secondary antibody.

2.5. Flow cytometry

Cell surface HER2 expressions of MDA-MB-231, MCF-7, SK-BR-3, BT-474, and SK-OV-3 and binding capability of T-CUS_{245C} were assessed by Flow cytometry on a FACS Calibur (BD Biosciences) flow cytometer. For HER2 expression, cells were incubated with FITC labeled trastuzumab (diluted to 1ng/105 cells) for 30 min at 4 °C and washed twice with PBS containing 1% FBS. For verifying T-CUS_{245C} binding ability, trastuzumab and T-CUS_{245C} were used respectively on SK-OV-3 and detected by anti-human-APC as a secondary antibody.

Each experiment was repeated triple times.

2.6. Cytotoxic activity

The dose- and time-dependent cytotoxic activity of drugs was measured by SRB assay as previously described [17]. Briefly, cells in logarithmic phase were seeded in 96-well plates with 3×10^4/ml per well and allowed to attach overnight before they were treated with CUS_{245C}, Trastuzumab, and T-CUS_{245C} for 3d and 5d, respectively. Colorimetry was performed at 515 nm with Epoch Microplate Reader (BioTek instrument, Inc). The dosage/time-effect curve was drawn using GraphPad Prism 5 (GraphPad Software). The cytotoxic activity was defined by IC_{50} values. Each experiment was repeated triple times.

2.7. Confocal microscopy

CUS_{245C} and T-CUS_{245C} we applied were visualized by *anti*-CUS_{245C} labeled with FITC using Fluorescein FITC Protein Labeling Kit (FITC, Thermo Fisher Scientific). Cells were seeded on glass coverslips in 6-well dishes at a density of 2×10^5 cells per well and allowed to attach overnight. To analyze the pathway of T-CUS_{245C} internalization cells were incubated with 1 ng/ml T-CUS_{245C} for 1 h, 4 h and 10 h respectively at 37 °C. After washed three times with PBS, cells were fixed and permeabilized and CUS_{245C} were detected by anti- CUS_{245C}-FITC. Lysosomes were further stained with specific dyes for lysosomes (Lyso-Tracker Red, Molecular probes), and nucleus were stained by Hoechst. Images were obtained by confocal microscopy using LSM 710 system (Carl Zeiss) with 63 × water *C*-Apochromat objective.

2.8. Mathematical and statistical analysis

The Coefficient drugs interaction between T-CUS_{245C} and propranolol, and T-CUS_{245C} and rolipram were identified by the results of CDI (Coefficient drugs interaction) formula: $CDI = AB/(A \times B)$. AB represents the survival rate of the combination of A and B, while A represents the survival rate of using A alone and B represents the survival rate of using B alone. CDI>1, antagonism; CDI = 1, additivity; CDI<1, synergism.

Experimental data were presented as mean ± SD and analyzed by SPSS 19.0. Two groups were analyzed by *t*-test. $P < 0.05$ was considered significantly different.

3. Results

3.1. T-CUS_{245C} construction

To determine whether combining trastuzumab with CUS would be practicable, we used an artificial disulfide bond to conjugate trastuzumab with recombinant CUS_{245C}. As shown in 6% SDS-PAGE (Fig. A), the conjugations were the mixtures of T-CUS_{245C}, T-PDP, CUS_{245C}, and CUS_{245C} dimers (Fig. A1 Lane 1). The residual of CUS_{245C} and T-PDP were removed by dialysis and Ni-NTA column successfully (Fig. A1 Lane 2). T-PDP were washed by 15 mM imidazole (Fig. A2 Lane 4–10). Purified T-CUS_{245C} was finally obtained (Fig. A4 Lane 1,4, and 5).

The molecular weight and purity of T-CUS_{245C} were analyzed by SDS-PAGE and Western blotting. Flow cytometry analysis showed an overlay of fluorescent value range of T-CUS_{245C} and trastuzumab, indicated that the binding capacity of T-CUS_{245C} was not impaired by CUS_{245C} conjugation (Fig. 1C).

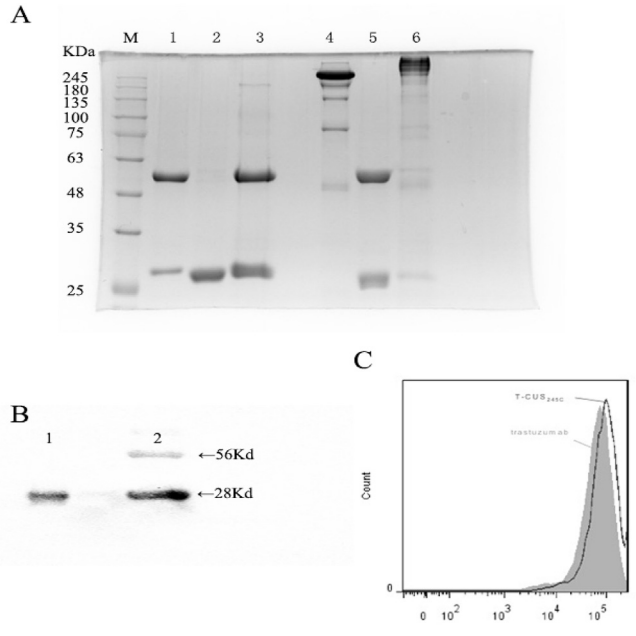

Fig. 1. Characterization of T-CUS_{245C}. A. SDS-PAGE showing the ladder of reduced trastuzumab (lane 1), reduced CUS_{245C}(lane 2), reduced T-CUS_{245C}(lane 3), unreduced trastuzumab (lane 4), unreduced CUS_{245C}(lane 5), and unreduced T-CUS_{245C}(lane 6), respectively; B. Western blotting testing the CUS_{245C} compartments on reduced CUS_{245C}(lane 1) and reduced T-CUS_{245C}(lane 2) using mouse *anti*-CUS_{245C} antibody and anti-mouse-HRP antibody; C. Affinity of T-CUS_{245C} compared with that of trastuzumab by flow cytometry.

3.2. Cytotoxicity of T-CUS_{245C} on different cell lines

To determine the cytotoxic effects of T-CUS_{245C}, we exposed antigen-positive cell lines mentioned above to CUS_{245C}, trastuzumab,T-CUS_{245C}, and T + CUS_{245C} for 3-day and 5-day treatments, respectively. SRB analysis were applied to determine living cells and OD_{515} values were later measured. Generally, the proliferation inhibition of T-CUS_{245C} on HER2 positive cells is dose-dependent and is remarkably more significant than that of trastuzumab, CUS_{245C}, and T + CUS_{245C} when their concentrations were less than 10^2nM. However, as concentration further increased, the cytotoxicity of CUS_{245C} emerged and rise dramatically (Fig. 2). This indicates that CUS_{245C} has limited cytotoxic activity at low concentration, but when it was conjugated to trastuzumab, it possessed a potent cell killing ability. For 3-day experiments, the IC_{50} of T-CUS_{245C} towards BT-474 and SK-BR-3 are above 0.1 nM but decreased to 0.07 ± 1.23 nM when it comes to SK-OV-3 (Table 1). The same cytotoxicity tendency can be observed in 5-day treatments, except the IC_{50} values decrease 10-fold to extremely low concentrations (Fig. 2B), illustrates a time-dependent cytotoxicity of T-CUS_{245C}. Although the cellular proliferation inhibition on day 3 is slightly mild for the T-CUS_{245C} treatment compared with the 5-day ones, its IC_{50} on SK-OV-3 is 10 times lower than T-DM1, an FDA approved trastuzumab-depend antibody drug conjugates (ADCs), with similar IC_{50} on other HER2 positive cells (Table 1). This demonstrates a potent in vitro anti-tumor efficacy of T-CUS_{245C} against HER2 positive cell lines, and among all cells been tested, SK-OV-3 might be the most sensitive one.

Interestingly, while the apoptosis of HER2 positive cells induced by trastuzumab and T + CUS_{245C} are not so distinct as T-CUS_{245C} did, they exerted anti-tumor efficacy on BT-474 when the concentrations beyond 0.1 nM (Fig. 2B), demonstrates that BT-474 is likely to be a trastuzumab-sensitive cell line even anti-tumor effectiveness of mediating antibody-dependent cell-mediated cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC) were absent. (Fig. 2).

To assess the cytotoxic specificity of T-CUS_{245C}, HER2 negative cells MDA-MB-231 and MCF-7 were also applied. The results in Fig. 2 and Table 1 showed that neither did MDA-MB-231 nor did MCF-7 react with trastuzumab, T + CUS_{245C} or T-CUS_{245C} ($IC_{50} > 1000$ ng/ml), confirming that the antigen binding is necessary for cytotoxic activity.

Subsequently, we test the extracellular level of HER2 on cells that we used. Mean florescent intensities (MFI) were acquired by flow cytometry and displayed on Fig. 2C. BT-474 has the highest HER2 expression level while SK-OV-3 and SK-BR-3 were relatively lower. MCF-7 and MDA-MB-231 were considered as HER2 negative cells. These data were strongly related with IC_{50} of T-CUS_{245C} showed in Table 1, indicates that the growth inhibition potency of ITs may not consistent with the level of the antigen expression.

3.3. Internalization of T-CUS_{245C}

To confirm the internalization and localization of T-CUS_{245C}, we applied confocal microscopy and used SK-OV-3 cells incubated with T-CUS_{245C} for various lengths of time up to 10 h to assess the internalization effects. Time-dependent internalization data (Fig. 3A) showed that the fluorescent signal was detected mainly on the cell surface treated with T-CUS_{245C} for 1 h at 37 °C. For 4 h, intense signals started to accumulate in cytoplasm. As incubation times extent to 10 h, no signal can be detected extracellularly while intracellular signal remained. Interestingly, when we used lysotrackers with red signal to make lysosomes visible, we found that green signal and red signal overlaid perfectly at 3-D position of cells after 4 h, meaning that ITs accumulated in lysosomes after they

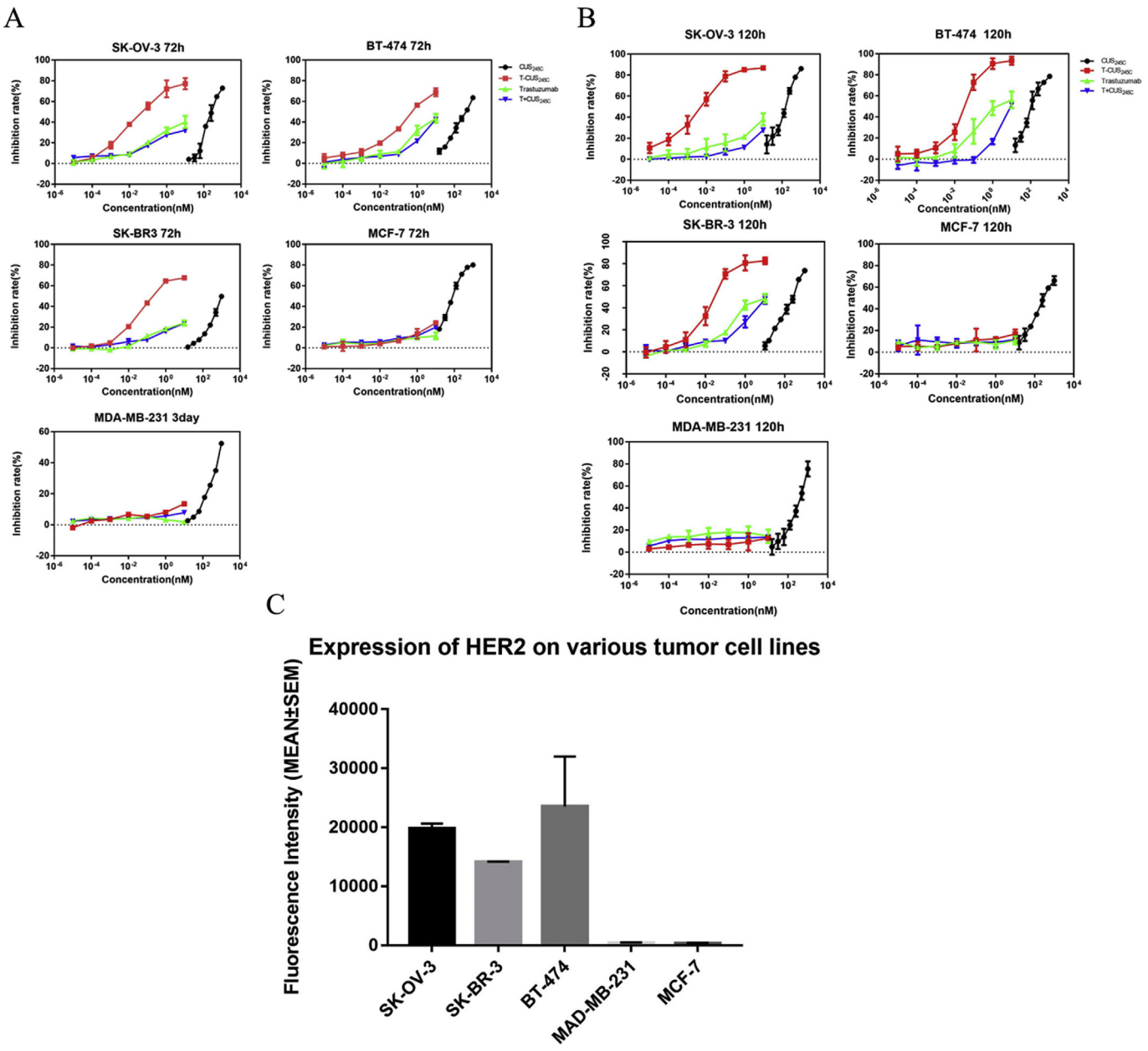

Fig. 2. Proliferation inhibition rates determined by SRB. A. Cytotoxicity of different drugs on different cells for 3 days assessed by SRB assay. B. Cytotoxicity of drugs on different cell lines for 5 days. C. HER2 expression on various cell lines.

Table 1
IC_{50} values of cells treated with drugs for 72 h and 120 h.

IC_{50}(nM,mean ± SD)					
Cell Lines	CUS_{245C}	T-CUS_{245C}	Trastuzumab	T + CUS_{245C}	T-DM1
SK-OV-3 (72 h)	287.20 ± 1.07	0.07 ± 1.23****	>10	>10	0.80 ± 0.03
SK-OV-3 (120 h)	143.80 ± 1.07	0.005 ± 1.23****	>10	>10	(−)
MCF-7 (72 h)	89.85 ± 1.05	>10	>10	>10	>66.67
MCF-7 (120 h)	315.90 ± 1.07	>10	>10	>10	(−)
BT-474 (72 h)	404.80 ± 1.05	0.67 ± 1.14****	>10	>10	0.77 ± 0.48
BT-474 (120 h)	127.90 ± 1.09	0.03 ± 1.18****	2.57 ± 1.40***	8.96 ± 1.14***	(−)
MDA-MB-231 (72 h)	934.90 ± 1.08	>10	>10	>10	(−)
MDA-MB-231 (120 h)	392.90 ± 1.08	>10	>10	>10	(−)
SK-BR-3 (72 h)	1016.00 ± 1.04	0.37 ± 1.23****	>10	>10	0.13 ± 0.07
SK-BR-3 (120 h)	235.70 ± 1.06	0.04 ± 1.27****	>10	>10	(−)

Data shown are IC_{50} values (mean ± SD, n > 3) of various tumor cells treated with drugs above for 72 h and 120 h respectively, measured by SRB assay; Compared with CUS_{245C}, $^{***}p < 0.001$,$^{****}p < 0.0001$.

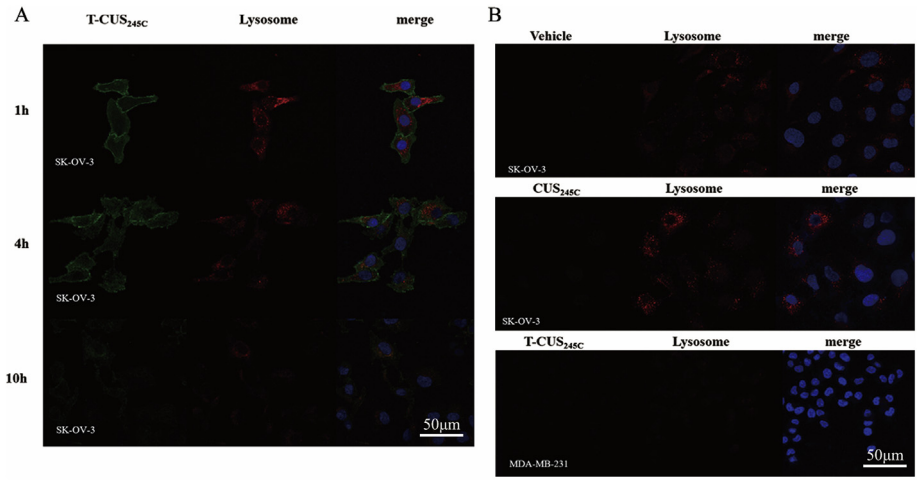

Fig. 3. Internalization of T-CUS_{245C} confirmed by confocal. A. internalized level of T-CUS_{245C} at 1 h, 4 h, and 10 h in SK-OV-3; B. PBS-treated, CUS_{245C}-treated SK-OV-3 cells and T-CUS_{245C}-treated MDA-MB-231 cells for 4 h; C. PBS or propranolol treated SK-OV-3 and BT-474 for 1 h. Scale bars 50 μm.

were internalized. Further, the internalization effects of T-CUS_{245C} were tested on HER2 negative MDA-MB-231 cells and neither did cell membrane nor cytoplasm had detectable fluorescence value (Fig. 3B). This data demonstrates that MDA-MB-231 cells can barely be delivered into cells as HER2 expressed is absent. Also, CUS_{245C} barely attached on cell surface for lacking binding moiety (Fig. 3B).

Subsequently, phosphatidic acid hydrolysis inhibitor, propranolol, which can trigger the endocytosis was administered to accelerate receptor accumulation and internalization of T-CUS_{245C}. As shown in Fig. 4A, when treated with 100 μM propranolol, T-CUS_{245C} can be detected in cytoplasm at 1 h, but no green fluorescence could be detected without propranolol's promotion or with combination effects of both 100 μM propranolol and 10 μM rolipram. This means propranolol facilitated the internalization of T-CUS_{245C} and allowed it entered cells faster while rolipram inhibited it. To test the relationship between internalization and cytotoxicity, propranolol and PDE4 inhibitor rolipram were applied to regulate the endocytosis of T-CUS_{245C} while SBR analysis was applied to evaluate the inhibition of proliferation of SK-OV-3. 100 μM of propranolol and 10 μM rolipram were choose according to the dose-dependent cytotoxicity of these two drugs for they were the maximum concentration with less cytotoxicity themselves (Fig. 4B). The 3-day combination treatments showed a significant increased or decreased cell killing effects of T-CUS_{245C} + propranolol or T-CUS_{245C} + rolipram than T-CUS_{245C} alone did, meaning the intense of cytotoxicity of T-CUS_{245C} correlation with internalization level positively to some extent, making internalization activators promising candidates for ITs combination therapies (Fig. 4C). Analyzing the inhibition rates, we calculate the CDI of both T-CUS_{245C} + propranolol and T-CUS_{245C} + rolipram using $CDI = AB/(A \times B)$ formula [18]. Table 2 showed a significantly synergistic effects of combining T-CUS_{245C} with propranolol. For T-CUS_{245C} + rolipram combination treatment, the CDI showed an antagonistic effect when the concentration of T-CUS_{245C} was at 0.1 nM. Although, the CDI of rolipram combined with 1 nM and 0.01 nM T-CUS_{245C} were slightly less than 1, the results showed no statistic significant difference.

Date shown are mean value of inhibition rate of drugs for SK-OV-3, $^{**}p < 0.05$, $^{***}p < 0.001$.$^{****}p < 0.0001$.

4. Discussion

Trastuzumab is a humanized monoclonal antibody against HER2/neu protein approved by FDA for treating breast and gastric cancer. However, majority of patients might become resistant during treatments and helping them retain sensitivity can be beneficial.

Type 1 RIPs are commonly used toxic payload of ITs. Our previous studies have shown a potent cytotoxicity of a type 1 RIPs CUS_{245C} and offered the evidences that it could be a perfect candidate for forming an IT [17,19]. Utilizing CUS_{245C} combining trastuzumab is likely a way of overcoming resistance issues via bypassing the signal pathway and killing cells directly. Here, we implemented chemical conjugation methods to fuse trastuzumab to CUS_{245C}, hoping that CUS_{245C} can overcome the limitation of trastuzumab and wider it's therapeutic window.

In this study, we proposed an alternative chemical conjugation strategy to obtain T-CUS_{245C} with a decent yield around 50% and almost 100% purity (Fig. A). In addition, no antigen binding affinity changed by extra CUS_{245C} conjugation (Fig. 1C).

In cytotoxicity study, the IC_{50} for SK-OV-3 is around 10pM, significantly lower than that of BT-474 and SK-BR-3. Whereas, HER2 expression on SK-OV-3 is similar with that of SK-BR-3 but less than that of BT-474, demonstrating that the cytotoxicity of IT is not severely correlated with antigen expression level and SK-OV-3 cells are most reactive one to T-CUS_{245C}. Temptingly, in comparison with other trastuzumab-based conjugates, such as trastuzumab-DM1, trastuzumab-maytansine, and anti-HER2-curcin, which IC_{50} values for SK-BR-3 is around 48.4 ng/ml (≈323pM), 40.5 ng/ml (≈270pM), and 4 nM, respectively [20], the antitumor efficacy of T-CUS_{245C} is extremely remarkable with IC_{50} ten times, even thousand times lower than trastuzumab-based conjugates mentioned above. Another interesting point is that apoptosis was barely induced in cells under trastuzumab stress, which means the trastuzumab-mediated effects were absent here, but it should not be underestimated in vivo since trastuzumab impart therapeutic benefit via ADCC and CDC when it is administered in vivo.

Many researches have studied the receptor-mediated endocytosis of ITs and found out that the internalization is through endocytosis and correlated with the cytolethal potency. Our confocal microscopy analysis showed that T-CUS_{245C} can be taken up in to endocytic vesicles as well. Moreover, neither did CUS_{245C} attach to cell nor did they be internalized into cytoplasm, illustrates that distinct internalization was enhanced mediated by antibodies. These results agree with what had been reported and ensure that our ITs exert their functions via a typical endocytosis pathway when ligands bind to their receptors. Importantly, not all the

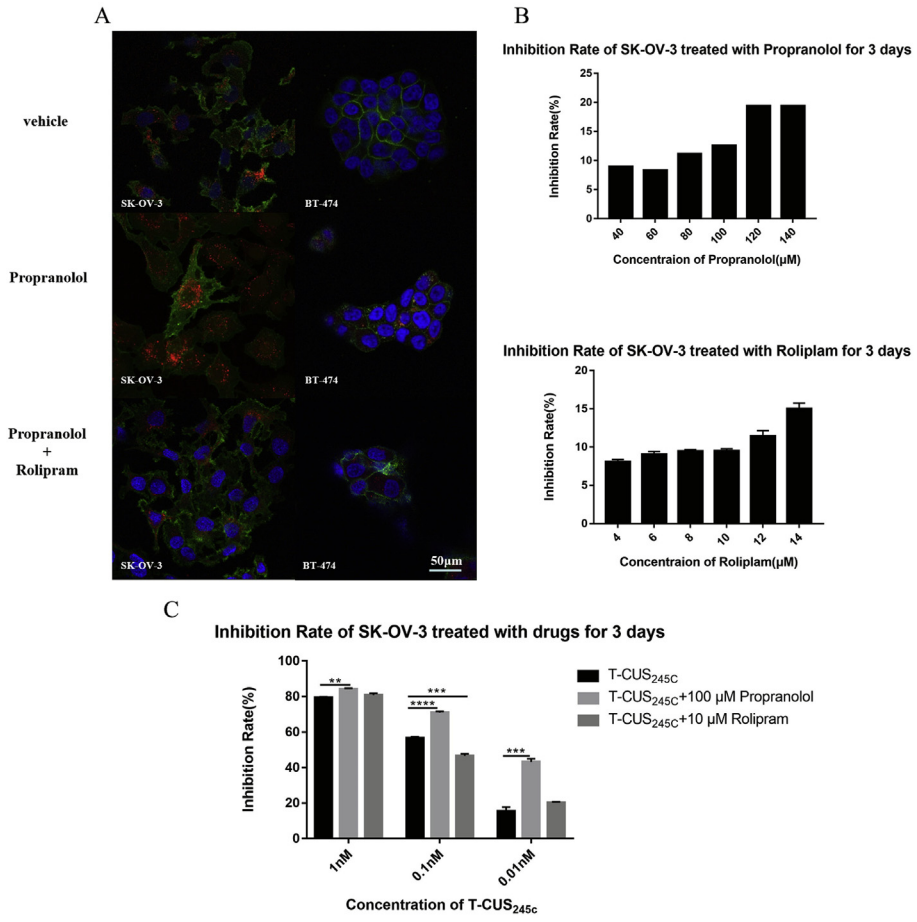

Fig. 4. The co-relationship of Internalization of T-CUS_{245C} with its cytotoxicity. A. The T-CUS_{245C} internalization of SK-OV-3 and BT-474 treated with propranolol and rolipram, respectively, for 1 h by confocal microscopy. B. The dose-dependent cytotoxicity of propranolol and rolipram, respectively. C. The cytotoxicity of T- CUS_{245C}, T-CUS_{245C} + Propranonlol, and T- CUS_{245C} + Rolipram for 3 days. Scale bars 50 μm.

Table 2
Coefficient drugs interaction of T-CUS_{245C} and propranolol or rolipram on SK-OV-3 for 3-day treatment.

T-CUS_{245C} nmol/L	Inhibition rate (%)			CDI	Interaction
	T-CUS_{245C}	100 μM Propranolol	T- CUS_{245C} +100 μM Propranololsss		
1 nM	79.29	15.41	84.10	0.65	$(+)^{**}$
0.1 nM	56.57		70.95	0.57	$(+)^{****}$
0.01 nM	15.46		43.21	0.57	$(++)^{***}$
T-CUS_{245C} nmol/L	Inhibition rate (%)			CDI	Interaction
	T-CUS_{245C}	10 μM Rolipram	T- CUS_{245C} +10 μM Rolipram		
1 nM	79.29	1.72	80.79	0.91	(+)
0.1 nM	56.57		46.66	1.201	$(-)^{****}$
0.01 nM	15.46		20.17	0.93	(+)

receptors expressed on cellular surface have the potential to be internalized, some cell membrane proteins are more likely to adjust their expression at post-translation level. Hence, understanding the mechanisms by which cells interact with environment and the role internalization plays in may be of great help to pick up targets suitable for IT treatment.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.03.090.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.03.090.

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