A new and simple non-chromatographic method for isolation of drug/linker constructs: vc-MMAE evaluation

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ABSTRACT

Introduction: Auristatin and its derivatives (synthetic analogues of dolastatin 10, an antineoplastic natural product), are highly potent antimitotic agents which have attracted considerable attention because of their cytotoxic activity when targeting tumor cells in the form of antibody-drug conjugates (ADCs). Some sophisticated and expensive equipment such as HPLC are needed for drug/linker isolation. The aim of this study was to provide a simple aqueous work-up procedure for the isolation of such drug/linker constructs. The anti-tumor activity of the extracted drug/linker was also investigated against SKBR3 and HEK293 cancer cell lines, and cell viability was assessed.

Methods: In the present study, monomethyl auristatin E (MMAE), a derivative of the cytotoxic tubulin modifier auristatin E, was covalently coupled to maleimidocaproyl-valine-citrulline-p-aminobenzoyloxycarbonyl (MC-vc-PAB), a cathepsin-B-cleavable linker, to obtain MC-vc-PAB-MMAE (vc-MMAE). Afterwards, a non-chromatographic isolation procedure was developed to isolate the drug/linker (vc-MMAE) construct. Silica gel thin-layer chromatography and electrospray ionization mass spectrometry were used to monitor the isolation procedure and to confirm the coupling of the linker to the drug, respectively. Further, the anti-tumor activity of the extracted drug/linker was investigated against SKBR3 and HEK293 cancer cell lines, and cell viability was assessed.

Results: After coupling, the isolation process was confirmed as a single spot on the TLC plate. The isolation yield was calculated to be 65%. [M + H]⁺, [M + 2Na]⁺ and [M + ACN + 2H]⁺ species were observed in the mass spectra, showing that the coupling of MMAE to the linker is not adversely affected by the workup method. Our data revealed that the isolated vc-MMAE was highly potent against tumor cell lines, exhibiting that the workup procedure did not affect MMAE-mediated cytotoxicity.

Conclusion: The isolation method described in this study can be applied for the development of a wide variety of ADCs.

Implication for health policy/practice/research/medical education:
vc-MMAE could be successfully isolated by the aqueous work-up method. The method described here can be potentially used for a variety of drug/linker isolations.


Introduction:
Dolastatins are natural cytotoxic pseudopeptides first isolated from the marine shellless mollusk Dolabella auricularia (1-3). Dolastatin 10 is one of the most potent tubulin polymerization inhibitors within the family (4), which shows potent activities against a wide range of lymphomas, leukemia and solid tumors in preclinical studies (5). In 1987, the structure of dolastatin 10 was elucidated by collecting mollusk biomass (1 ton) from the wild to isolate just 29 mg of dolastatin 10. Two years later, a convergent total synthesis of dolastatin 10 could resolve any resupply issues (6). However, dolastatin 10
has failed to enter the clinical trials as a single agent for cancer therapy due to its off-target and systemic toxicity (7,8). To circumvent this issue, an attractive approach is to conjugate such highly potent cytotoxic drugs to tumor-specific mAbs, known as antibody-drug conjugates (ADCs). Fundamentally, ADCs exert the specificity of tumor-specific mAbs, which often lack therapeutic activity, to deliver highly potent cytotoxic molecules selectively to cancer cells, resulting in both improved potency of the mAbs and decreased undesirable systemic toxicity of the cytotoxic molecules (7-9). Tremendous efforts have led to the synthetic development of the potent dolastatin 10 analog, monomethyl auristatin E (MMAE) (10-12). This cytotoxic molecule similarly inhibits cell division by blocking the tubulin polymerization (13-15).

MMAE is as much as 100- to 1000-fold more potent than the standard chemotherapeutic drugs such as vinblastine and, therefore, cannot be used as a drug in its current form (7,8). Because MMAE is totally synthetic, it is possible to incorporate its functional groups for mAb attachment through a variety of conjugation strategies (10,11,16-18). Both ADC components (drug and antibody) are conjugated by a linker, a component that plays a key role in ADC stability. An ideal linker should be stable in systemic circulation to minimize toxicity but allows rapid release of active free drug inside tumor cells (19).

Recent studies demonstrated that the protease-labile dipeptide linker maleimidocaproyl-valine-citrulline-p-aminobenzoyloxy-carbonyl (MC-VC-PAB) is much more stable in vivo than other cleavable linkers (11,20). To date, the MC-vc-PAB linker coupled to the drug MMAE (vc-MMAE) has been widely used to develop various ADCs. vc-MMAE-based ADCs, as one of the leading types, has been widely utilized in clinical trials (21), such as Glembatumumab (phase II) (22) and PSMA-ADC (phase I) (23). More recently, the US FDA has approved a vc-MMAE-containing ADC, brentuximab vedotin (Adcetris®), to treat CD30-positive Hodgkin lymphoma and systemic anaplastic large-cell lymphoma (24).

The majority of ADCs utilize attachment of the linker-drug directly to interchain cysteine residues of the antibody (25). Therefore, to develop such an ADC, the drug-linker construct is often produced by chemical methods and then isolated using chromatography, followed by conjugation with a reduced mAb (11,13,26). However, chromatographic methods are more expensive and time-consuming which require more skilled personnel (27). Therefore, there is a need for a simple and cost-effective method capable of effectively purifying such drug/linker constructs.

In the present study, after attachment of the drug MMAE to the linker MC-vc-PAB, the resultant vc-MMAE construct was isolated by a simple non-chromatographic method, so-called workup. Silica gel thin-layer chromatography was used to monitor the isolation procedure. Afterwards, the coupling of MMAE to the linker was analyzed using electrospray ionization mass spectrometry (ESI-MS), and the in vitro cytotoxicity of the construct was measured using the MTT assay. Herein, we report a simple and efficient workup method that can be utilized for the isolation of vc-MMAE constructs that retain measurable cytotoxicity against tumor cell lines.

Materials and methods
Attachment of MC-vc-PAB linker to MMAE
An MC-vc-PAB linker (Concertis Biosystems, Corp., San Diego, CA, USA) was coupled to the N-terminal amino acid of MMAE (Concertis Biosystems, Corp., San Diego, CA, USA) to form vc-MMAE, according to the method of Francisco et al and Doronina et al (11,13) with minor modification. Briefly, the MC-vc-PAB linker (26 mg, 0.0352 mmol, 1.5 equiv.) was dissolved in a minimum amount of N,N-dimethylformamide (DMF; Sigma-Aldrich, Germany), followed by gentle stirring at room temperature (RT) for one hour. Disopropylethylamine (DIPEA; 18.39 µL, 0.1056 mmol, 3 equiv; Merck, Darmstadt, Germany), as a base to promote the reaction, was added to the mixture. N,N,N′,N′-Tetramethyl-O-(benzotriazol-1-yl) uronium tetrafluoroborate (TBTU; 33.8 mg, 0.1056 mmol, 3 equiv.; Sigma-Aldrich, Germany), as an uronium coupling reagent, and hydroxybenzotriazole (HOBT; 5.7 mg, 0.0423 mmol, 1.5 equiv.; Chem-Impex, Wood Dale, IL, USA), as an activating reagent to yield the intermediates, were added to the solution with continuous stirring at RT for 30 minutes. Subsequently, the drug MMAE (16.9 mg, 0.0235 mmol, 1 equiv.) was added to the mixture and incubated for 48 hours at RT under continuous stirring to form a dark brown solution.

Isolation of vc-MMAE (workup method)
To isolate the vc-MMAE construct, the solution from the previous step was added dropwise to a beaker containing 20 mL distilled water with continuous stirring. The process was repeated until the construct was completely isolated. Immediately after isolation, the dark brown solution changed to yellow, yielding a white precipitate. The resulting mixture was allowed to stir for 30 minutes at RT. Finally, an off-white precipitate of vc-MMAE was recovered by vacuum filtration, and stored at -80°C until used.

Thin layer chromatography
The isolation progress was monitored by Thin layer chromatography (TLC). TLC was performed on 10 x 5 cm pre-coated silica gel plate (aluminium sheets, silica gel 60 F254 plates of Merck KGaA, Germany). All solvents and reagents used were of analytical grade obtained from Merck. The length of the chromatogram run was 8 cm.

Apparatus and chromatographic conditions
The present study used an Agilent 6410 triple quadrupole mass spectrometer (Agilent technologies, USA) equipped
with an electrospray ionization interface (ESI) coupled to an Agilent 1200 HPLC (Agilent Technologies, USA).

**Liquid chromatography**

Samples were injected using Liquid chromatography (LC) without column (through union) controlled by Mass-Hunter workstation software ver. B.04.00. A connector was used instead of the column to allow direct injection of samples. The mobile phase consisted of a mixture of MeOH/H2O (90:10, v/v) + 0.1% formic acid, at ambient temperature and a flow rate of 0.5 mL/min. Total run time was 7 minutes.

**Mass spectrometry**

An Agilent 6410 triple-quadrupole mass spectrometer interfaced with ESI ion source was used to detect analytes. Optimal MS parameters were as follows: Drying gas (nitrogen) flow rate: 6 L/min, Nebulizing gas adjusted at 15 psi, capillary voltage of 4.0 kV, and desolvation temperature of 300°C. Nitrogen was used as the desolvation gas. The data were processed using the MassHunter software. Detection was performed in full scan mode by an ESI source operating in the positive ionization mode (ESI+). The fragmentor voltage was 60 V. The source temperature was set to 350°C and ion spray voltage was 4.5 kV.

**Cell lines and culture**

SkBR3 (human breast cancer cell line) and HEK-293 (human embryonic kidney cell line) were obtained from National Cell Bank of Iran (Pasteur Institute of Iran, Tehran, Iran). The cells were cultured in high-glucose Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich) supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 unit/mL penicillin, 100 μg/mL streptomycin and 0.2 mM Glotamax (all from Invitrogen Gibco), and incubated at 37°C in a 5% CO2 atmosphere. At about 80% confluence, the cells were detached by trypsin-EDTA (Sigma, St Louis, MO), and cell numbers were counted with a hemocytometer. Cell viability was recognized specifically by a self-immolative spacer, para-amino benzyloxycarbonyl (PABC) and MMAE. Protease-cleavable dipeptide linkers are coupled to the N-terminal position of MMAE through a self-immolative p-aminobenzylcarbamate spacer (PABC) (11). The presence of a maleimidocaproyl (MC) spacer provides enough room so that the vc group can be recognized specifically by a tumor-associated lysosomal enzyme cathepsin B that cleaves the citrulline-PABC amide bond. The resultant PAB-substituted MMAE is an unstable intermediate that spontaneously undergoes a 1,6-elimination with a loss of p-iminoquinonimethide and carbon dioxide (self-immolation), leaving MMAE as the final product (26,29).

**Cell cytotoxicity assay**

SkBr3 and HEK293 cells were seeded into 96-well tissue culture plates (Greiner, Frickenhausen, Germany) at an initial density of 1.5×104 and 1×104 cells/well, respectively. The plating density and assay time course were optimized for each cell line. At 80% confluency, the cells were treated with the MC-vc-PAB linker and vc-MMAE construct at different concentrations (0.01024, 0.00512, 0.0256, 1.28, 6.4, 32, 160, 800, 4000 nM) in triplicate. Cells with no treatment were used as a negative control. After 72-hour incubation, the medium was aspirated, and the formazan crystals in cells were dissolved in 100 μL of dimethyl sulphoxide (DMSO, Sigma Aldrich, USA). Subsequently, the plates were incubated on a rotary shaker at 37°C for one hour to solubilize the formations of purple crystal formazan, and the absorbance was measured at 570 nm. The absorbance of untreated control cells was considered to be 100% survival. The cytotoxicity rate was calculated using the following formula: cytotoxicity (%) = 100 - ((At-Ab)/(Ac-Ab)) × 100, where At = Absorbance value of the test compound, Ab = Absorbance value of the blank and Ac = Absorbance value of the negative control. The IC50 (half maximal inhibitory concentration) value was estimated as a concentration causing 50% cell death with respect to the control.

**Statistical analysis**

Data were expressed as mean ± standard deviation (SD) of the mean of at least three independent experiments. Statistical significance was determined using a multiple comparison t test. P values less than 0.05 were considered to be statistically significant.

**Results**

**Structure of the vc-MMAE construct**

In the present study, the drug MMAE was coupled to the linker MC-vc-PAB to generate vc-MMAE. The vc-MMAE construct consists of a thiol-reactive maleimide-containing linker attached to the drug MMAE. As illustrated in Figure 1, the vc-MMAE construct utilizes a protease-sensitive dipeptide, valine-citrulline (vc), a self-immolative spacer, para-amino benzyloxycarbonyl (PABC) and MMAE. Protease-cleavable dipeptide linkers are coupled to the N-terminal position of MMAE through a self-immolative p-aminobenzylcarbamate spacer (PABC) (11). The presence of a maleimidocaproyl (MC) spacer provides enough room so that the vc group can be recognized specifically by a tumor-associated lysosomal enzyme cathepsin B that cleaves the citrulline-PABC amide bond. The resultant PAB-substituted MMAE is an unstable intermediate that spontaneously undergoes a 1,6-elimination with a loss of p-iminoquinonimethide and carbon dioxide (self-immolation), leaving MMAE as the final product (26,29).

**Isolation and characterization of the vc-MMAE construct**

In the present study, a simple aqueous work-up procedure was used for the isolation of vc-MMAE. The isolation process was monitored by TLC. Results from TLC revealed that all the starting material disappeared, giving rise to a single spot (data not shown). After isolation, the product (off-white powder) was weighed and the yield of the process was estimated to be 65%. ESI-MS was used to confirm the drug/linker coupling. Results from ESI-MS analysis showed the formation of the vc-MMAE construct. [M + H]+, [M + 2Na]+ and [M + ACN + 2H]+ species were
detected in the positive ion mass spectra (data not shown).

**Cytotoxic effects of vc-MMAE on the proliferation of human cancer cell lines**

To assess whether the functionality of the vc-MMAE construct was compromised by the workup method, we examined the biological activity of MC-vc-PAB linker and vc-MMAE on SKBR3 and HEK293 cells in a dose-response experiment (Figures 2 and 3). Cells were treated for 72 hours with the vc-MMAE and MC-vc-PAB linker, as a negative control, at concentrations ranging from 0.01024 nM to 4 µM. As shown in Figures 2A and 3A, microscopic observations revealed that vc-MMAE has the ability to induce cell death in both SkBr3 and HEK293 cell lines, as compared to cells treated with the MC-vc-PAB linker. The MTT assay was used to determine whether the vc-MMAE construct has a cytotoxic effect on the cell lines. Results showed that vc-MMAE could induce cell death in the SKBR3 cancer cells at concentrations ranging from 0.01024 nM to 4 µM, as compared with the MC-vc-PAB linker (Figure 2B). vc-MMAE exhibited a significant increase in cell death at a concentration of 4 µM (69.11% ± 4.04) compared to other concentrations (P < 0.0001). A concentration of vc-MMAE showing 50% reduction in cell viability (IC50 value) was calculated to be 410.54 ± 4.9 nM for SKBR3 cell population. The cytotoxic activities of vc-MMAE and MC-vc-PAB were also examined on the HEK293 cell line. As illustrated in Figure 3B, vc-MMAE exhibited a significant cytotoxic activity against the HEK293 cancer cell line at concentrations ranging from 0.01024 nM to 4 µM, compared to the MC-vc-PAB linker. The vc-MMAE construct displayed significantly increased cell death at a concentration of 4 µM (65.935% ± 3.24) compared to other concentrations (P < 0.0001). Dose-
response experiments showed that the IC50 value of the vc-MMAE construct was 482.86 ± 6.4 nM in HEK293 cell population. The ability of vc-MMAE to kill SKBR3 and HEK293 cell lines obviously increased at concentrations 32 to 4000 nM, representing about two-fold more cytotoxic compared to concentrations lower than 32 nM.

Discussion
The majority of ADCs utilize attachment of linker-drug constructs directly to interchain cysteine residues of antibodies (26). To develop such ADCs, the drug-linker construct is frequently produced using chemical methods, isolated, and then conjugated to reduced mAbs (11,13,20,26). Chromatography is commonly used method for the isolation of drug/linker constructs (11,13). In this study, we described a simple non-chromatographic method for the isolation of vc-MMAE. In a study carried out by Francisco et al (13), a reverse-phase preparative-HPLC (RP-HPLC) column was used to isolate the vc-MMAE construct, providing an extraction yield of 70%. Dorolina et al (11) could purify maleimidoacryloyl-peptide-MMAE derivatives and maleimidoacryloyl-AEVB using C18 reverse-phase preparative HPLC with a yield of 57%. In addition, Lyon et al (30) also used RP-HPLC for the isolation of several drug/linker constructs, including Maleimido-DPR-val-cit-PAB-MMAE (48% yield), Maleimido-Dab-val-cit-PAB-MMAE (9% yield), Maleimido-Orn-val-cit-PAB-MMAE (11% yield), Maleimido-Lys-val-cit-PAB-MMAE (39% yield) and Maleimidoacetyl-val-cit-PAB-MMAE. Chromatography-based methods, although considered to be sensitive and reliable, require sophisticated equipment, skilled analysts, and time-consuming sample preparation steps. In addition, organic solvents used in the detection process may result in environmental pollution (27). Therefore, establishment of simple, inexpensive and non-chromatographic separation methods capable of efficiently isolating drug/linker constructs can be a valuable alternative for ADC development. In our previous studies, we used a simple method for the isolation of some small molecules, such as cyclopeptides containing triazole skeleton (31), proline-rich heptapeptide (32) and protected enkephalins (33). Our isolation method is a simple aqueous procedure (workup) that is able to remove reagents and by-products generated in the reaction. After coupling, the isolation process was confirmed as a single spot on the TLC plate. Consistent with the above studies, we could isolate the vc-MMAE with a yield of 65%, highlighting the effectiveness of this method for vc-MMAE isolation. After coupling the drug to the linker, ESI-MS was used to characterize the structure of linker/drug construct, showing that the linker was coupled to the drug in a correct manner. Results from ESI-MS analysis demonstrated that our isolation method did not affect the structure of vc-MMAE. The vc-MMAE construct isolated by this method was tested for biological activity. The MTT colorimetric assay was performed to determine the cytotoxic activity of vc-MMAE against SkBr3 and HEK293 cell lines. In light of this, the cytotoxicity effects of various concentrations of the MC-vc-PAB linker and vc-MMAE construct were assessed in both cell lines and compared with untreated cells. Results showed that the vc-MMAE construct could significantly diminish cell viability of SKBR3 and HEK293 with IC50 values of 410.54 ± 4.9 and 482.86 ± 6.4 nM, respectively. Consistent with our findings, Burns et al (34) demonstrated that MMAE coupled to a succinimide nitrophenyl-based linker could significantly induce cell death in HeLa and MDA-MB-231 cell lines in a dose-dependent manner. In another study, Szlachcic et al (35) tested the potency of vc-MMAE on different cell lines, demonstrating that vc-MMAE has IC50 values of 480, 560, 580 and 520 nM for BJ, U2OS, U2OS-FGFR1 and MDA-MB-134-V1 cell lines, respectively. Our bioassay results are consistent with these findings, highlighting that our isolation method did not affect the functionality of the drug/linker construct. As the present vc-MMAE showed a significant antitumor effect at low concentrations, we move toward to use the vc-MMAE for the development of a promising ADC. MMAE-based ADCs display potent antitumor activities by inhibiting the tubulin polymerization in dividing cells (9,11,13,36,37). A wide variety of studies showed that vc-MMAE-conjugated mAbs are quite stable under physiological conditions but undergo rapid hydrolysis in tumor cells, leading to cell death (11,13,37).

Conclusion
In summary, we demonstrated that vc-MMAE could be isolated by a more convenient method than the commonly used chromatographic techniques. The method described here can potentially provide a promising avenue for conjugation chemistry. However, the effectiveness of this construct for ADC synthesis remains to be elucidated.

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Authors’ contributions
All authors contributed to the study. MAA conducted the experiment, analyzed and discussed results, wrote conclusion and prepared the manuscript for publication. MHA, SB, and MA designed the study. ML analyzed data and discussed results. FG also conducted the experiment and analyzed the results. All authors read and approved the final manuscript.

Conflict of interests
The authors declared no competing interests.

Ethical considerations
Authors have entirely regarded ethical issues such
as plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy.

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