Original Article

Monomethyl Auristatin E, a Potent Cytotoxic Payload for Development of Antibody-Drug Conjugates against Breast Cancer

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Abstract

Background: Breast cancer is a heterogeneous disease characterized by differential responses to targeted and chemotherapeutic agents. Antibody-drug conjugates are one of the promising strategies for the treatment of breast cancer. Monomethyl auristatin E (MMAE) is a highly potent microtubule inhibitor and a common payload used for development of antibody-drug conjugates. The purpose of this study was to investigate the cytotoxic effects of MMAE on breast cancer cell lines.

Materials and Methods: MDA-MB-468 and MDA-MB-453 cells were treated with MMAE at various concentrations (1, 10, 100, and 1000 ng/ml), and cytotoxicity was measured after 48 and 72 hours using an MTT assay.

Results: Our findings indicated that MMAE possesses dose- and time-dependent cytotoxic activities against human breast cancer cells. The morphological features of the treated cells were supportive of the cytotoxic activity of MMAE. The results of the MTT assay showed that MMAE has a significant cytotoxicity against MDA-MB-468 and, to a lesser degree, MDA-MB-453 cells.

Conclusion: MMAE can be used as a highly cytotoxic payload for development of antibody-drug conjugates against breast cancer.

Keywords: Monomethyl auristatin E, cytotoxicity, Antibody-drug conjugate, MDA-MB-468, MDA-MB-453

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Introduction

Breast cancer is one of the most commonly diagnosed malignancies and the leading cause of cancer-related deaths in women worldwide[1], accounting for an estimated 1 million new diagnoses and 400,000 deaths per year[2]. Several chemotherapeutic drugs have been approved for the treatment of breast cancer, but effective treatment remains elusive[3]. Monoclonal antibodies (mAbs) against antigens expressed on cancer cells have been considered as an alternative option to traditional cancer chemotherapy. Trastuzumab (Herceptin®), a humanized IgG1 mAb, is currently used for the treatment of breast cancer, specifically for human epidermal growth factor receptor 2 (HER2)-positive breast cancer. However, 70% of the patients with HER2-positive breast

cancers stop responding or lose clinical benefits by primary (denovo) or secondary (acquired) resistance[4].

Over the past years, significant efforts have been devoted to improve the therapeutic activity of mAbs through various modifications. One promising approach has been to incorporate antibodies and cytotoxic drugs (payloads) in a single molecular entity, known as antibody-drug conjugates (ADCs)[5]. ADCs combine the targeting advantages of mAbs with the cytotoxic potential of small-molecule payloads to enhance specific drug delivery in tumor cells[5-11]. Cytotoxic drugs used for ADCs must meet three requirements, including strong cell toxicity, the presence of a functional group suitable for the coupling to the antibody, and a definite mechanism of action[12]. There are a lot of cellular toxins known in nature, but only a small number of toxic agents have been found to be suitable for ADC applications.

The payloads currently being used in ADC development can generally be divided into two main categories, microtubule inhibitors and DNA-damaging agents[13]. The former includes auristatins and maytansines that target rapidly dividing cells by interfering with different parts of the cell cycle[14] whereas the latter comprises calicheamicins, along with the less commonly used cytotoxins such as duocarymycins and pyrrolobenzodiazepine (PBD) dimers, that induce DNA damage[15].

Monomethyl auristatin E (MMAE), a highly potent auristatin, remains a hot topic in ADC studies. MMAE is an antimitotic agent which inhibits cell division by blocking the polymerisation of tubulin, representing as much as 100- to 1000-fold more potent than standard chemotherapeutic drugs such as vinblastine[16,17]. The drug is currently used as a payload for development of a variety of ADCs, including Brentuximab vedotin (Adcetis®, SGN-35), an FDA-approved ADC, and more than fourteen ADCs in different phases of clinical trials. More importantly, MMAE are extensively used as a payload for development of ADCs against breast cancer, including Glembatumomab vedotin (CDX-011, phase II) and SGN-LIV1A (phase I)[16]. Determination of MMAE cytotoxic potential in breast cancer cell lines may increase the chances of successful ADCs for the treatment of breast cancer.

In the present study, we sought to investigate the anticancer activity of MMAE in two kinds of breast cancer cell lines, MDA-MB-468 and MDA-MB-453. The cells were exposed to various concentrations of MMAE and the growth response was measured in a dose- and time-dependent manner. Then, cell Cytotoxicity was determined using the MTT assay.

Methods

Drug preparation: MMAE was bought from Concortis (San Diego, USA). Defined MMAE solutions were prepared, filtered using 0.22-µM-pore-size filters and serially diluted with the medium to obtain working solutions. Lastly, the working solutions were stored at −20˚C until the time of the experiment.

Cell Lines: MDA-MB-468 and MDA-MB-453 (human breast cancer cell lines) were obtained from national cell bank of Iran (Pasteur Institute of Iran, Tehran, Iran). Cells were grown in DMEM medium supplemented with 10% heat inactivated FBS, 100 unit/ml penicillin, 100 µg/ml streptomycin and 0.2 mM Glotamax (Invitrogen Gibco), under an atmosphere of 95% humidity and 5% CO2 at 37°C. Cells were maintained by sub-culturing and passaging as monolayers in 25- and 75-cm_2 cell culture flasks.

Cell Culture: For the experiments, cells were detached by trypsin-EDTA (Sigma-Aldrich; St. Louis, MO, USA) at approximately 80% confluency and cultured in 96-well tissue culture plates (Greiner, Frickenhausen, Germany) at a density of 12×10^3 cells/well[18]. After 24 hours of incubation, the cells were exposed to MMAE at various concentrations (1, 10, 100, and 1000 ng/ml) for the next 48 and 72 hours. Cells treated with no drug or DMSO were used as negative and positive controls, respectively. All experiments were repeated five times. Each experiment was performed in duplicate under the same conditions.

Trypan Blue Dye Exclusion Assay: Cell suspension was mixed with an equal volume of 0.4% trypan blue reagent (Gibco Life Technologies), the solution was mixed thoroughly and the number of viable and nonviable cells was determined by trypan blue dye exclusion using a hemocytometer. The number of live cells was calculated using the following formula: % viability = (live cell count/total cell count)*100.

Cellular Morphology: The effect of MMAE on cell morphology was determined using an inverted light microscope (Leica, Inc.) during 48- and 72-hour incubation periods. Once digital images were recorded,

cells were trypsinized and resuspended in PBS containing 1% FBS for further analyses.

Cytotoxicity assay: An MTT assay (Sigma-Aldrich; St. Louis, MO, USA) was used to assess the *in vitro* cytotoxicity of MMAE. After 48- and 72-hour incubation periods, the medium was aspirated, and cells were washed twice with PBS. Afterwards, 20 µl/well MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) solution (stock concentration, 5 mg/mL in PBS) was added to each well, and incubated for 4 hours in an incubator at 37°C. After the incubation period, the media was gently aspirated, and the formazan crystals in cells were dissolved in 200 µl of DMSO. The plates were incubated on a rotary shaker at 37°C for 1 hour to solubilize the formations of purple crystal formazan. The absorbance was measured using a microplate reader at 570 nm. The absorbance of untreated cells was considered to be 100% survival. The cytotoxicity rate was determined using the following formula: cytotoxicity (%) = 100 - ((At-Ab)/ (Ac-Ab)) × 100, where At = Absorbance value of the drug, Ab= Absorbance value of the blank and Ac=Absorbance value of the negative control.

Statistical analysis: Statistical analyses were carried out using GraphPad Prism version 6.00. Data were presented as mean ± standard deviation (SD) of the mean of at least three independent experiments. Two-way ANOVA and multiple comparison t test were used to calculate the statistical significance. *p*-values less than 0.05 were considered to be statistically significant.

Results

In the present study, the *in vitro* cytotoxic activity of MMAE was assessed against breast cancer cell lines in a dose- and time-dependent manner. Before the experiment, the viability of the cells was assessed by the trypan blue dye exclusion test; fundamentally, viable cells with an intact cellular membrane lack the ability to take up the dye (a clear appearance) while damaged nonviable cells take up dye through their membrane damages (a blue appearance). Cell viability was estimated to be approximately 95%.

MDA-MB-468 and MDA-MB-453 cell lines were treated for 48 and 72 hours with MMAE at various concentrations (1, 10, 100, and 1000 ng/ml). Cells with no treatment were used as a negative control. The morphological changes of MMAE-treated cells were compared with the untreated cells. As illustrated in Figure 1, MMAE-treated cells exhibited a significantly altered morphology under the inverted microscope. Cells treated with MMAE, specifically at higher concentrations, appeared less uniform with the loss of membrane integrity, showing significant differences with untreated cells. Remarkable changes include loss of the intact membrane, cell detachment from the plate, loss of contact with neighboring cells, cytoplasmic condensation, and cell shrinkage as compared to untreated cells[18].

MTT was used to determine the cytotoxic effects of MMAE on breast cell lines. As shown in Figure 2, MMAE exhibited a significant cytotoxic activity against MDA-MB-468 and MDA-MB-453 cells at concentrations ranging from 1 to 1000 ng/ml, as compared to the untreated cells.

The highest cytotoxicity ($p \leq 0.0001$) was found when MDA-MB-468 (Figure 2A) and MDA-MB-453 (Figure 2B) cells were treated with MMAE at a concentration of 1 µg/ml for 72 hours, showing inhibition rates of 62.98 % ± 5.03 and 49.455 ± 2.9, respectively.

MDA-MB-468 cells treated with MMAE exhibited no significant differences between 48- and 72-hour periods at the same concentration, except for a concentration of 1ng/ml (Figure 2A, $p \leq 0.05$). In contrast, significant differences were found between different time periods in MDA-MB-453 cells (Figure 2B, $p \leq 0.001$). However, MMAE could induce a dose-dependent increase in cytotoxicity in both cell lines.

For further investigation, the cytotoxic activity of MMAE was compared in the two cell lines. As depicted in Figure 3, MMAE exhibits a higher cytotoxic effect on MDA-MB-468 cells when compared to the MDA-MB-453 cells. Broadly speaking, the MDA-MB-468 cell line was shown to be more sensitive to MMAE than the MDA-MB-453 cell line in time- and dose-dependent manners. Additionally, lower concentrations of MMAE showed more increased cell death in MDA-MB-468 as compared to MDA-MB-453.

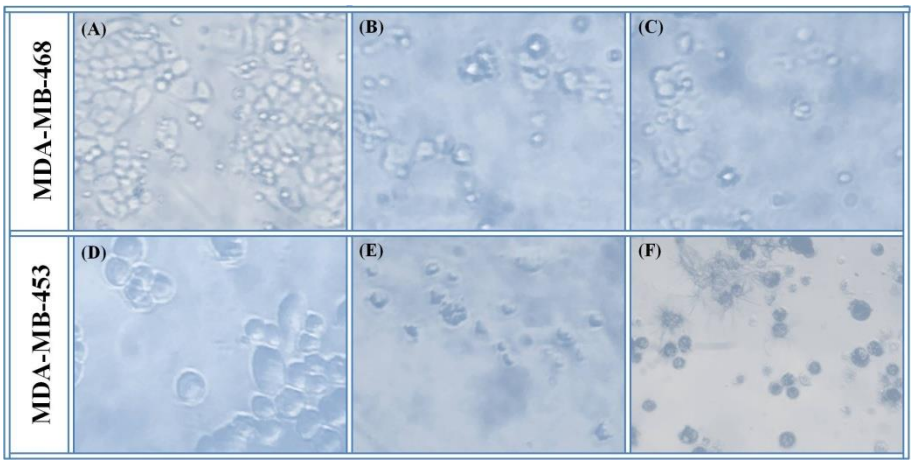

Figure 1. Morphological features of MDA-MB-468 and MDA-MB-453 cells after 48- and 72-hour treatment with Monomethyl auristatin E (MMAE). MDA-MB-468 and MDA-MB-453 cells were treated with different concentrations (1, 10, 100, and 1000 ng/ml) of MMAE, and morphological changes were determined using an inverted light microscope. Significant differences were found between controls and cells treated with MMAE. The upper panel indicates MDA-MB-468 cells with no (A), 48-hour (B), and 72-hour (C) treatment. The lower panel represents MDA-MB-453 cells with no (D), 48-hour (E) and 72-hour (F) treatment. Cells exposed to MMAE showed loss of intact membrane, loss of contact with neighbouring cells, and detachment from the culture plate.

Discussion

In this study, we demonstrated the cytotoxicty activity of MMAE against two kinds of breast cancer cell lines. Results from our study showed that MMAE is highly cytotoxic against breast human cancer cell lines MDA-MB-468 and MDA-MB-453 at nanomolar concentrations. However, it was found that MDA-MB-468 is more sensitive than MDA-MB-453 in both time- and dose-dependent manners. As shown in Figure 3, MMAE induced more cell cytotoxicity in MDA-MB-468 than that in MDA-MB-453. MDA-MB-468 (ER-, PR-, HER2-) was found to be more sensitive than MDA-MB-453 (ER-, PR-, HER2+). Whereas the role and mechanisms of HER2 overexpression on chemosensitivity still require intensive investigation, findings from a variety of studies suggest that HER2 overexpression results in increased chemoresistance to certain chemotherapeutic agents. A variety of studies demonstrated that HER2-overexpressing breast cancer cells are more resistant to certain chemotherapeutic agents, as compared with HER2-nonoverexpressing breast cancer cells[19]. Yu D et al. showed that higher expression of HER2 in human breast cancer cell lines expressing HER2 correlated with resistance to paclitaxel, and downregulation of HER2 using an anti-HER2 mAb significantly sensitized the cell lines to the drug[20]. Their results revealed that HER2 overexpression renders human breast cancer cells resistant to paclitaxel[19]. In another study, Sellappan S et al. revealed that a human breast cancer cell MDA-MB-435 stably transfected with the HER2 gene showed no change in the expression of the multidrug resistance gene (MDR), but the HER2-overexpressing transfectants were more resistant to paclitaxel and docetaxel than their parental cells[21]. Additionally, several studies have demonstrated that overexpression of ABCC3 (ATP-Binding Cassette Subfamily C Member 3) plays an important role in acquired multi drug resistant (MDR) in cancer cell lines[22,23]. Notably, Burns KE et al. showed that stable overexpression of ABCC3 results in in vitro resistance to MMAE[24]. They suggested that the ABCC3 amplicon was most commonly associated with the HER2-amplified subtypes. Nevertheless, our results showed that both cell lines are potentially sensitive to MMAE, when compared to untreated cells. We demonstrated that MMAE has the ability to induce cell death in MDA-MB-468 and MDA-MB-453 cells; up to 60% cell growth inhibition was observed in cells treated with 1 µg/ml of MMAE, whereas no significant inhibition of proliferation was detected in untreated cells.

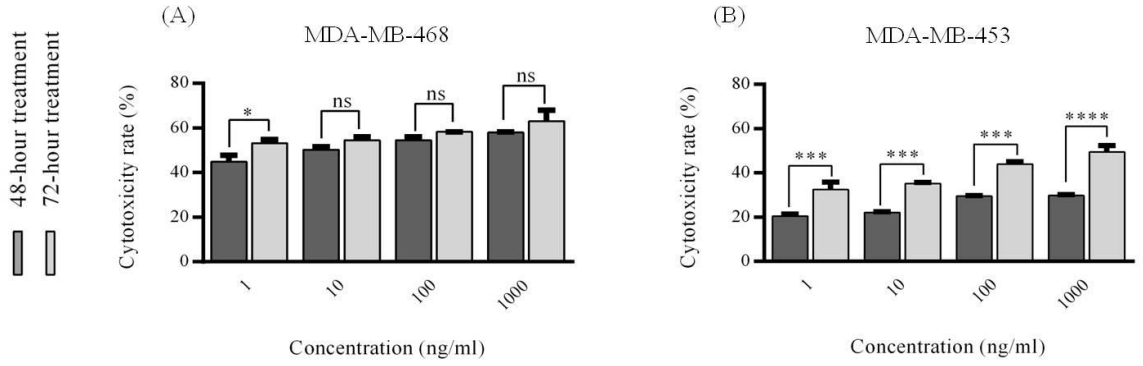

Figure 2. Effects of Monomethyl auristatin E (MMAE) on the proliferation of MDA-MB-468 (A) and MDA-MB-453 (B) cells after 48- and 72-hour treatment with Monomethyl auristatin E (MMAE). Different concentrations (1, 10, 100, and 1000 ng/ml) of MMAE were assessed on the cell lines, and the cytotoxcity rate was measured using the MTT assay after a 48- and 72-hour exposure period, as described in "Materials and Methods". The data represent the mean and the error bars indicate standard deviation (SD) of two independent experiments (ns: P > 0.05, *: P ≤ 0.05, **: P ≤ 0.01, ***: P ≤ 0.001, and ****: P ≤ 0.0001).

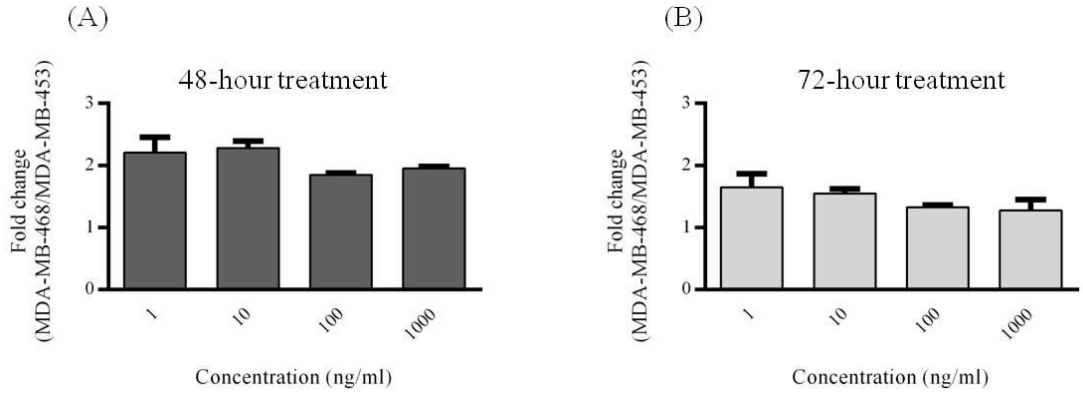

Figure 3. Comparison of the cytotoxic activity of Monomethyl auristatin E (MMAE) on MDA-MB-468 and MDA-MB-453 cell lines. The percentages for the cell cytotoxicity of MMAE-treated MDA-MB-468 cells were normalized to that of MDA-MB-453 cells. After normalization, the effect of MMAE on the two cell lines was compared.

MMAE is a synthetic antineoplastic agent, which cannot be used as a drug itself due to its high toxicity; instead, it is conjugate to a mAb to form ADCs. Although under investigation for decades, ADCs have received renewed attention with the advent of significant advances in engineering new linker and conjugation technologies together with highly potent cytotoxic drugs. The most commonly used toxins for ADCs in clinical development are maytansine, calicheamicin, and auristatin derivatives[25].

In the present study, we selected the highly potent tubulin inhibitors MMAE because this agent is well characterized and suitable for modification to facilitate coupling to an antibody[25]. To date, the drug MMAE has been widely used to construct various ADCs. The MMAE-based ADCs used in the clinic trials include Glembatumumab vedotin (phase II)[26] and PSMA-ADC (phase I)[27]. Lately, the US FDA has approved a vc-MMAE-containing ADC, brentuximab vedotin (Adcetris®), for the treatment of CD30-positive Hodgkin lymphoma and systemic anaplastic large-cell lymphoma[28].

Conclusion

MMAE represents potent antitumor activities against breast cancer cells, highlighting its great potential for the development of ADCs against breast cancer.

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