

Original Article

Monomethyl auristatin E Exhibits Potent Cytotoxic Activity against Human Cancer Cell Lines SKBR3 and HEK293

Meghdad Abdollahpour-Alitappeh¹, Sepand Razavi-vakhshourpour¹, Majid Lotfinia², Saeed Jahandideh², Hamid Najminejad¹, Saeed Balalaie³, Reza Moazzami⁴, Elnaz Shams⁴, Mahdi Habibi-Anbouhi⁵, Mohsen Abolhassani^{1*}

¹Hybridoma Laboratory, Immunology Department, Pasteur Institute of Iran, Tehran, Iran

²Department of Biochemistry, Pasteur Institute of Iran, Tehran, Iran

³Peptide Chemistry Research Group, K.N. Toosi University of Technology, Tehran, Iran

⁴Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran

⁵National Cell Bank of Iran, Pasteur Institute of Iran, Tehran, Iran

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Abstract

Background: Monomethyl auristatin E (MMAE) is a synthetic analog of dolastatin 10, a compound originally isolated from the marine mollusk. MMAE, as a highly potent microtubule inhibitor, exerts its potent cytotoxic effect by inhibiting microtubule assembly, tubulin-dependent GTP hydrolysis and microtubules polymerization. This molecule, by itself, lacks the tumor specificity required to elicit therapeutic benefit. Nevertheless, the extremely cytotoxic potential of MMAE could be harnessed in the form of MMAE-antibody conjugates. The aim of the present study was to evaluate the cytotoxic activity of MMAE against breast (SKBR3) and kidney (HEK293) cancer cell lines in an in vitro cell-based assay.

Materials and Methods: SKBR3 and HEK293 cells were treated with different concentrations ranging from 0.002048, 0.01024, 0.0512, 0.256, 1.28, 6.4, 32, 160, 800 and 4000 nM of MMAE, and cell viability was determined after 72 hours using an MTT colorimetric assay. The effect of MMAE was regularly monitored by direct observation using an invert microscope.

Results: Microscopic observation showed that there was a concentration-dependent increase in cell death. Results from the MTT assay revealed a statistically significant loss of viability ($P < 0.0001$) at concentrations ranging from 0.01024 to 4000 nM in SKBR3 cells, and 0.0512 to 4000 nM in HEK293 cells. Our findings showed that MMAE inhibited the growth of SKBR3 and HEK293 cells in a concentration-dependent manner, with IC₅₀ values of 3.27 ± 0.42 and 4.24 ± 0.37 nM, respectively.

Conclusion: MMAE was able to significantly inhibit cell growth at nanomolar concentrations, emphasizing its great potential for the development of antibody-drug conjugates.

Keywords: Monomethyl auristatin E; cytotoxicity; Antibody-drug conjugate; SKBR3; HEK293

*Corresponding Author: Mohsen Abolhassani; Hybridoma Lab, Immunology Department, Pasteur Institute of Iran, Tehran, Iran; Tel. and Fax: (+98) 21-66492596. Email: mabolhassani@yahoo.com

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Introduction

Dolastatins are natural cytotoxic pseudopeptides, which were first isolated from the small Indian Ocean sea hare *Dollabella auricularia*. The dolastatin family includes cemadotin, tasidotin (ILX651), soblidotin, malevamide E and dolastatin 10^{1,2}. Dolastatin 10 has demonstrated potent activity in preclinical studies against a range of lymphomas, leukemia and solid tumors^{3,4}. As a potent disruptor of tubulin polymerization⁵, dolastatin 10 inhibits the binding of Vinca alkaloids to tubulin in a noncompetitive fashion, and stabilize the binding of colchicines to tubulin. This potent antitumor agent, in addition to tubulin polymerization inhibition, has a strong inhibitory effect on tubulin dependent GTP hydrolysis⁶, which can serve as an extremely potent mitotic spindle poison⁷. Dolastatin 10 consists of a four amino acid peptide (dolavaline, valine, dolaisoleuine, dolaproine) linked to a complex primary amine (dolaphenine), showing a potent cytotoxic activity at nanomolar concentrations⁵. Although the dolaisoleuine amino acid residue is critical for the inhibition of tubulin polymerization, the dolaproine or dolavaline amino acids can be modified without compromising the function of the molecule^{8,9}.

Because it is difficult to extract large quantities of dolastatin 10, efforts have resulted in the development of the potent synthetic dolastatin 10 analogs, termed auristatin, including Monomethyl auristatin F (MMAF) and Monomethyl auristatin E (MMAE). MMAE is a highly potent auristatin (free drug IC₅₀: 10⁻¹¹-10⁻⁹ M) developed by Seattle Genetics. This cytotoxic molecule represents as much as 100 to 1000 folds more potent than the standard chemotherapeutic drugs such as vinblastine^{10,11}. However, MMAE, as well as MMAF, is currently being used as payloads in antibody-drug conjugates (ADCs), showing excellent antitumor activities in the clinic such as Glematumumab and PSMA-ADC^{10,12-17}. Importantly, an MMAE-antibody conjugate brentuximab vedotin (Adcetris®) has already been approved by the US Food and Drug Administration (FDA) for clinical use¹⁸.

Some studies evaluated the cytotoxic activity of auristatnes on a diverse panel of human tumor cell

lines including hematological malignancies, melanoma, and carcinomas of the lung, stomach, prostate, ovaries, pancreas, breast, colon and kidneys, and compared with the activities of another antimitotic agent, vinblastine, as well as to doxorubicin^{19,20}. However, there are no reports specifically examining the effect of MMAE on SKBR3 and HEK293 cell lines. Therefore, we sought to investigate the anticancer activity of MMAE and to determine the minimum effective dose in SKBR3 and HEK293 cells using an in vitro model. In the present study, the cells were exposed to various concentrations of MMAE and the growth response was measured in a dose-dependent manner. Cell viability was determined using the MTT assay. The findings from this study demonstrated potent cytotoxic activity of MMAE on human cancer cell lines SKBR3 and HEK293.

Methods

Cell Lines and Culture: SkBR3 (human breast cancer cell line) and HEK-293 (human embryonic kidney cell line) were purchased from National Cell Bank of Iran (Pasteur Institute of Iran, Tehran, Iran). 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 (Invitrogen Gibco), with 5% CO₂ and 95% humidity at 37°C. At approximately 80% confluency, the cells were detached by trypsin-EDTA (Sigma, St Louis, MO) and cell numbers were counted using a Neubauer hemocytometer²¹. Cell viability was estimated to be 95%.

MMAE preparation: MMAE was purchased from Concartis Biosystems (Corp, San Diego, CA, USA). The drug was stored at -20 until used. The solutions of MMAE were prepared and vigorously stirred before dilution in the filtered cell culture medium and stored at -20°C. The stock solution was then serially diluted with the medium to obtain working solutions. The drug concentration range used in this study was approximately the same as that reported in literature²².

Cell cytotoxicity assay: In vitro cell cytotoxicity was determined using an MTT assay. Cell suspensions containing 1.5×10⁴ and 1×10⁴ viable SkBr3 and

HEK293 cells, respectively, were cultivated in 96-well tissue culture plates (Greiner, Frickenhausen, Germany) with or without the drug MMAE in a final volume of 200 μ l, as triplicates. The plating density and assay time course were optimized for each cell line. MMAE was added approximately 24 hours after cell seeding. At 80% confluency, the cells were treated with MMAE at various concentrations (0.002048, 0.01024, 0.0512, 0.256, 1.28, 6.4, 32, 160, 800 and 4000 nM). Untreated cells were used as a negative control. After a 72-hour incubation, the medium was aspirated, cells were washed twice with PBS, and 100 μ l/well MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) solution (Sigma-Aldrich, 5 mg/mL in PBS) was added to each well; the cells were then incubated for 4 hours at 37°C. After the incubation period, the media was aspirated, and the formazan crystals in cells were dissolved in 200 μ l of dimethyl sulphoxide (DMSO, Sigma Aldrich, USA). Afterwards, 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 a wavelength of 570 nm. The absorbance of untreated cells was considered to be 100% survival. The cytotoxicity rate was determined using the following formula: cytotoxicity (%) = $100 - ((At - Ab) / (Ac - Ab)) \times 100$, where At = Absorbance value of the test compound, Ab = Absorbance value of the blank and Ac = Absorbance value of the negative control.

Statistical analysis: Statistical analyses and graphical representation of data were carried out using GraphPad Prism version 6.00 (GraphPad Software). Data were presented as mean \pm standard deviation (SD) of the mean of at least three independent experiments. Statistical significance was calculated using a multiple comparison t test. *P*-values less than 0.05

were considered to be statistically significant.

Results

In the present study, a dose-response experiment was performed to measure the in vitro cytotoxic activity of MMAE against SKBR3 and HEK293 cells (Figure 1). The cells were treated for 72 hours with MMAE at concentrations ranging from 0.002048 to 4000 nM. Cells with no treatment were used as negative controls. As shown in Figures 2A and 3A, microscopic observations demonstrated that MMAE was able to significantly induce cell death in both SkBr3 and HEK293 cell lines, compared to untreated cells.

MTT was used to determine the cytotoxic effects of MMAE on breast and kidney cell lines. As shown in Figure 2B, proliferation of SKBR3 cells was significantly inhibited by MMAE at concentrations ranging from 0.01024 to 4000 nM, as compared with cells with no treatment. MMAE exhibited a significant increase in cell death ($p < 0.0001$) at a concentration of 4 μ M with an inhibition rate of $88.81\% \pm 4.38$. A concentration of MMAE showing 50% reduction in cell viability (IC₅₀ [half maximal inhibitory concentration] values) was calculated to be 3.27 ± 0.42 nM in SKBR3 cell population. The effect of MMAE was also investigated in the HEK293 cell line. As illustrated in Figure 3B, MMAE was also able to significantly inhibit HEK293 cell proliferation at concentrations ranging from 0.0512 to 4000 nM, compared with untreated cells. MMAE displayed significantly increased cell death at a concentration of 4 μ M ($88.79\% \pm 2.61$), as compared with untreated cells ($p < 0.0001$). Dose-response experiments showed that the IC₅₀ value of MMAE was 4.24 ± 0.37 nM in HEK293 cell population. The ability of MMAE to kill both SKBR3 and HEK293 cell lines significantly

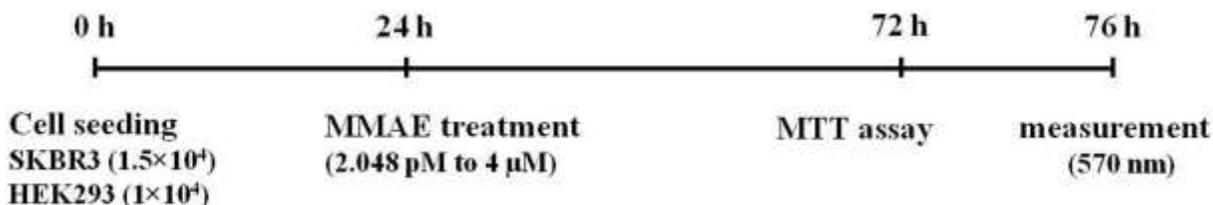


Figure 1. Schematic diagram of the experimental procedure to study the cytotoxic potential of MMAE on SKBR3 and HEK293 cell lines. After cell seeding, cells were treated with various concentrations of the drug. Following a 72-hour incubation, the MTT assay was carried out to evaluate cell viability in the presence and absence of the drug.

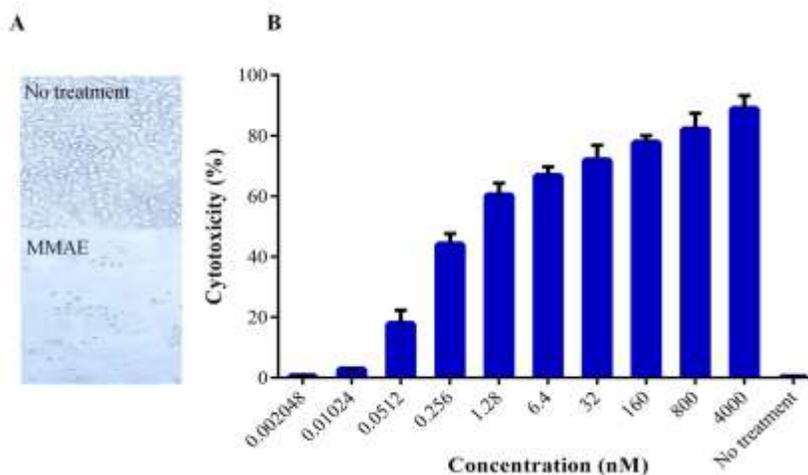


Figure 2. Effects of MMAE on the proliferation of the SKBR3 cell line. A) Microscopic observation was performed during the treatment. The upper and lower panels indicate cells receiving no treatment or treated with MMAE (6.4 nM), respectively. B) Cell viability was measured using the MTT assay after a 72-hour exposure period. Different concentrations (2.048 pM to 4 μM) of MMAE were assessed on the cell line, and the cytotoxicity rate was calculated as described in “Materials and Methods”. The data represent the mean and the error bars indicate standard deviation (SD) of three independent experiments.

increased when compared with untreated cells ($p < 0.0001$). The concentrations producing 50% growth inhibition potentially suppressed proliferation of SKBr3 and HEK293 cells.

Further investigation showed that MMAE, although displaying a significant cytotoxic potential against both cell lines, exhibits a higher cytotoxic effect on SKBR3 cells when compared with the HEK293 cell line, as depicted in Figure 4. The SKBR3 cell line was

demonstrated to be more sensitive to MMAE cell killing than the HEK293 cell line. Our findings revealed that SKBR3 had a significantly increased cell death rate at concentrations of 0.256 ($P < 0.05$) and 1.28 ($P < 0.01$) nM, when compared with that of HEK293. In addition, lower concentrations of MMAE showed more increased cell death in SKBR3 as compared with HEK293 (Figures 2 and 3).

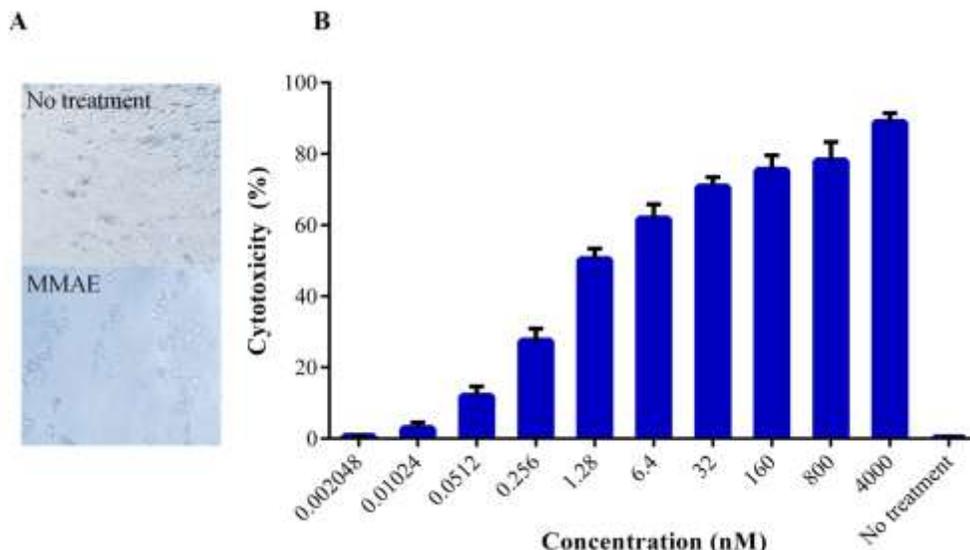


Figure 3. The effects of MMAE on the proliferation of the HEK293 cell line. A) Microscopic observation was carried out during the treatment. The upper and lower panels represent cells receiving no treatment or treated with MMAE (6.4 nM), respectively. B) Cell viability was evaluated using the MTT assay after a 72-hour exposure period. Different concentrations (2.048 pM to 4 μM) of MMAE were assessed on the cell line, and the cytotoxicity rate was calculated as described in “Materials and Methods”. The data represent the mean and the error bars indicate standard deviation (SD) of three independent experiments.

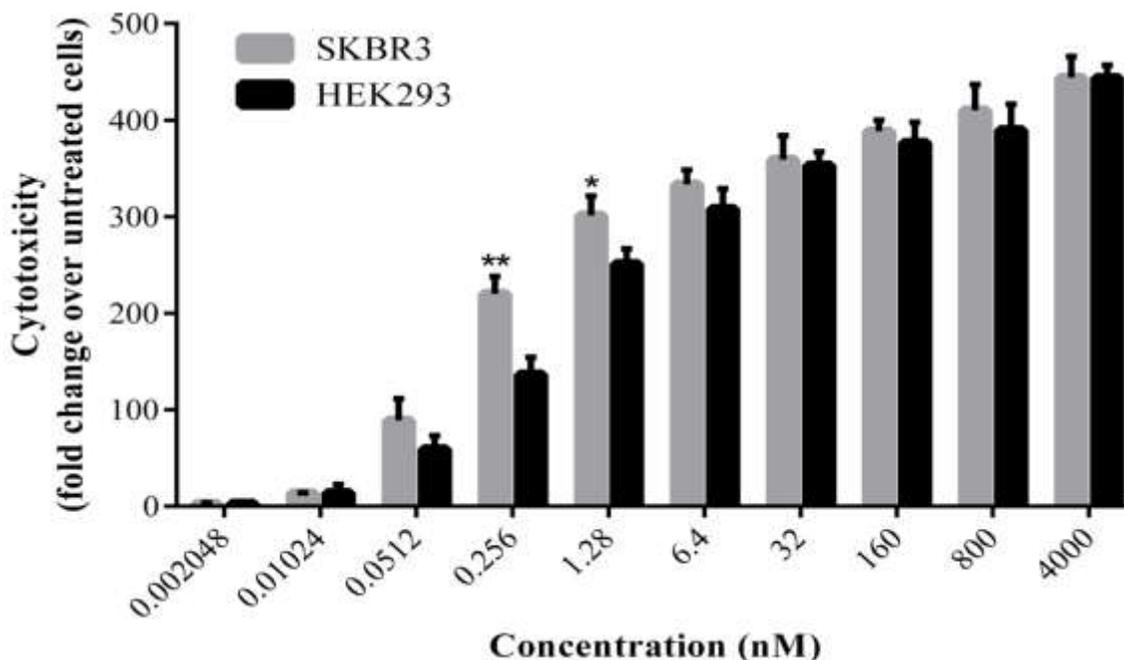


Figure 4. Comparison of the cytotoxic activity of MMAE on SKBR3 and HEK293 cell lines. The percentages for the cell viability of MMAE-treated cells were normalized to that of untreated control cells. After normalization, the effect of MMAE on the two cell lines was compared (* = $P \leq 0.05$, and ** = $P \leq 0.01$).

Discussion

In the present study, we investigated the cytotoxicity activity of MMAE, a synthetic antineoplastic agent, on two kinds of cancer cell lines. Results from our study showed that MMAE is highly cytotoxic against human cancer cell lines at nanomolar concentrations, consistent with our previous study on MDA-MB-468 and MDA-MB-453 cells²³. We also assessed the IC₅₀ of MMAE for SKBR3 and HEK293 cell lines, showing that MMAE has a higher IC₅₀ value for HEK293 compared with SKBR3. There are no reports to date specifically assessing MMAE effects on SKBR3 and HEK293 cell lines. However, some studies assessed the effect of MMAE, in addition to dolastatin 10 and auristatin E, on a variety of cancer cell lines^{19,20,22}. In a study carried out in 1990, Kalemkerian GP et al. showed that dolastatin 10 has potent growth inhibitory activity against four SCLC (small cell lung cancer) cell lines (NCI-H69, -H82, -H446, -H510) with IC₅₀ values ranging from 0.032 to 0.184 nM³. A study conducted on a panel of seven human lymphoma cell lines demonstrated that the average IC₅₀ value of auristatin E was 1.4 nM^{19,20}. In

2015, Burns KE et al. demonstrated that MMAE has potent cytotoxic activity against the HeLa cell with IC₅₀ value of 4nM. In addition, they reported that MDA-MB-231 cells might be resistant to MMAE when compared with HeLa cells²⁴. In 2016, Li H et al. showed that MMAE has a potent antitumor activity on NCI-N87Li H and BGC-823 cell lines, exhibiting IC₅₀ values of 7.7 and 9.1 ng/ml²². Consistent with above-mentioned studies, we demonstrated that MMAE has the ability to induce cell death in SKBR3 and HEK293. However, HEK293 seems to be less sensitive to MMAE treatment, as shown by lower cytotoxicity. We found that HEK293 has a higher IC₅₀ value (4.24 ± 0.37 nM) compared with SKBR3 (3.27 ± 0.42 nM). This may be due to the higher resistance of HEK293 cells to MMAE as compared with that of SKBR3 cells (Figure 4), because of upregulation of multidrug resistance (MDR) genes, key components of drug resistance in multiple cancers. In agreement with our findings, O'Brien C et al. demonstrated the resistance to MMAE in some cell lines. They showed that cell lines with the basal-like gene expression profile had lower average IC₅₀ values and were more sensitive to MMAE than luminal or HER2-amplified cell lines. Overexpression

of ABCC3 (ATP binding cassette subfamily C member 3) was reported to be the main cause of in vitro MDR²⁵. In 2015, Chen R et al.²⁶ reported that MMAE resistance might be due to altered intracellular accumulation of MMAE after drug internalization. In fact, MMAE can be actively pumped out of the cell by P-glycoprotein or other transporters. However, auristatins have an IC₅₀ that is 52- and 197-fold more toxic than that of the clinically relevant chemotherapies, such as vinblastine and doxorubicin, respectively^{13,24}, representing ultra-potent cytotoxic microtubule inhibitors that are clinically used as payloads in ADCs²⁷. MMAE, as a synthetic analog, contains a secondary amine at its N-terminus that could be attached to a linker and subsequently conjugated to monoclonal antibodies¹³. Although this potent drug is currently being used as payloads in ADC synthesis^{13,15,16,28}, determining cell sensitivity or resistance of tumor cell lines to MMAE can help design more highly selective targeted therapeutics. To date, the drug MMAE has been widely used to construct various ADCs. The MMAE-based ADCs has been widely utilized in the clinic trials²⁹, such as Glembatumumab (phase II)¹⁶ and PSMA-ADC (phase I)¹⁵. 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 lymphoma²⁸.

Conclusion

It is well established that a wide variety of small molecules applying in ADC development represent significant cytotoxic and chemopreventive properties. The present study provides evidence that in-vitro cytotoxic activity of MMAE dose-dependently inhibits the proliferation of SKBR3 and HEK293 cell lines. However, further studies on primary cultures are a valuable alternative to clarify the cytotoxic activity of MMAE.

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