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Synthesized Anti-HER2 Trastuzumab-MCC-DM1 Conjugate: An Evaluation of Efficacy and Cytotoxicity

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ABSTRACT

Background: Trastuzumab is a humanized monoclonal antibody that targets site-specifically human epidermal growth factor-2 receptor (HER2) cell surface antigen overexpressed in approximately 20% of human breast carcinomas. Despite its positive therapeutic outcomes, a large proportion of individuals are unresponsive to the treatment with the trastuzumab or develop resistance to it.

Objective: To evaluate a chemically synthesized trastuzumab-based antibody-drug conjugate (ADC) to improve the trastuzumab therapeutic index.

Methods: The current study explored the physiochemical characteristics of the trastuzumab conjugated to a cytotoxic chemotherapy agent DM1 via Succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) linker, created in our earlier study, using SDS-PAGE, UV/VIS, and RP-HPLC analyses. The antitumor effects of the ADCs were analyzed using MDA-MB-231 (HER2-negative) and SK-BR-3 (HER2-positive) cell lines utilizing in vitro cytotoxicity, viability, and binding assays. Three different formats of a HER2-targeting agent: trastuzumab, synthesized trastuzumab-MCC-DM1, and commercially available drug T-DM1 (Kadcyla®) were compared.

Results: UV-VIS spectroscopic analysis showed that the trastuzumab-MCC-DM1 conjugates, on average, entailed 2.9 DM1 payloads per trastuzumab. A free drug level of 2.5% was determined by RP-HPLC. The conjugate appeared as two bands on a reducing SDS-PAGE gel. MTT viability assay showed that conjugating trastuzumab with DM1 significantly improved the antiproliferative effects of this antibody in vitro. Importantly, the evaluations using LDH release and cell apoptosis assays confirmed that trastuzumab maintains its ability to induce cell death response while conjugating with the DM1. The binding efficiency of trastuzumab-MCC-DM1 was comparable to that of the naked trastuzumab.

Conclusion: Trastuzumab-MCC-DM1 was found effective against HER2+ tumors. The potency of this synthesized conjugate brings it closer to the commercially available T-DM1.

Keywords: Breast Neoplasms, Immunoconjugates, Therapeutic Index, Trastuzumab

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INTRODUCTION

According to the World Health Organization's (WHO) latest cancer statistics, approximately 7.8 million breast cancer cases were identified between 2015 - 2020, with 2.3 million diagnosed within just one year, 2020, accounting for the majority of the world's cancer cases (1, 2). A dramatically lower survival outcome along with a higher incidence of tumor aggressiveness and recurrence has been reported for nearly 15 – 20% of all breast cancer patients. Such clinical features are associated closely with cell surface antigen human epidermal growth factor receptor 2 (HER2) overexpression or gene amplification in this patient population (3). Functionally, the overexpression of this receptor triggers the formation of HER2 homo and heterodimers. This, in turn, promotes aberrant constitutive activation of pro-tumorigenic signaling cascades inducing unrestrained tumor growth (4-6). Thus, HER2 has become a major point of focus as a potential target for therapeutic interventions. In this context, a number of novel antibody-based therapies have been developed as key players in the field of breast cancer treatment, capable of specifically targeting the extracellular domain of HER2 and thus blocking the receptor dimerization as well as disrupting subsequent HER2-driven downstream signaling pathways (7-9). One such putative therapeutic agent is humanized monoclonal antibody trastuzumab (Herceptin®), the first HER2-targeted cancer immunotherapy to be approved, exhibiting favorable efficacy in treating breast cancer subtype with HER2 positivity (10, 11). However, despite the promising therapeutic outcomes, trastuzumab therapy, in itself, is associated with drawbacks of a modest response rate coinciding with a high incidence of resistance acquisition (12). More importantly, while trastuzumab is highly effective at targeting tumor cells, its lack of sufficient cytotoxicity typically results in only limited antitumor effects (13). In response to the mentioned concerns that highlight the need for innovative, more robust HER2-targeted therapies, trastuzumab selectivity has been leveraged in conjunction with the cytotoxicity of drug payloads. The result has been a new version of trastuzumab-based cancer therapy, addressing the poor effectiveness of trastuzumab in killing tumor cells and the toxicity of chemotherapy for healthy tissues (14). Known as antibody-drug conjugates (ADCs), these promising therapeutics harness the inhibitory effects of both the antibody and the cytotoxic agent, thus potentially ameliorating patients' clinical outcomes (15). To date, twelve ADCs targeting a range of malignancies have been granted the U.S. Food and Drug Administration's (FDA) approval, seven of which were approved as of 2019, and more than 80 potential late-stage investigational candidates are currently undergoing clinical trials worldwide (16, 17).

Trastuzumab-DM1 ADC (marketed as Kadcyla®) was the first member of this particular group of biotherapeutics to be approved in 2013 and shown to effectively overcome many mechanisms of trastuzumab resistance (15, 18, 19). It is employed as the standard of care in the second-line setting to offer hope of defeating primary or acquired resistance to trastuzumab, improving the therapeutic index, and bringing overall survival benefits to patients whose breast cancer has metastasized or advanced locally (20, 21). The trastuzumab-DM1 contains trastuzumab connected to the cytotoxic antimicrotubule chemotherapy agent DM1, a derivative of maytansinoid, via a bifunctional linker, SMCC (9). Indeed, trastuzumab is basically exploited as a transport mechanism for site-specific DM1 drug delivery to tumor cells expressing the HER2 target antigen. The antitumor activity of trastuzumab-DM1 primarily occurs through its target-dependent cellular internalization, thereby enabling selective cytotoxicity in HER2-expressing cancer cells while considerably reducing systemic side effects (9). The trastuzumab-DM1 acts independently of HER2-driven signaling and often needs only a high abundance of HER2

on the cellular surface (22).

Current research provides an in-house-prepared antibody-drug conjugate, trastuzumab-MCC-DM1, designed to target breast cancer with HER2-overexpression, representing an efficacy comparable to commercially available trastuzumab-based ADC, T-DM1. As a preliminary step toward claiming biosimilarity in a future application for marketing authorization from Iranian regulatory authorities, we performed comparative analyses between the two mentioned agents. Several in vitro analyses were conducted utilizing HER2-negative and HER2-positive breast cancer cell lines to determine whether this conjugate could effectively suppress breast cancer growth. To this end, physical/chemical quality attributes including drug-to-antibody ratio, free drug content as well as the pre and post-conjugation difference in the molecule size, and in vitro cellular activity of the trastuzumab-MCC-DM1 conjugate were compared with T-DM1, a control ADC created through similar conjugation chemistry, as well as unconjugated trastuzumab.

MATERIALS AND METHODS

Reagents

The Trastuzumab was from Aryogen Pharmed (Tehran, Iran) and T-DM1 was purchased from Genentech Inc. (South San Francisco, CA). MTT (3-(4, 5-dimethylthiazol-2-yl)-2–5-diphenyltetrazolium bromide) reagent and dimethylsulfoxide (DMSO) were obtained from Sigma-Aldrich (Saint Louis, MO). The FITC-conjugated Annexin V/7-Aminoactinomycin D (7-AAD) apoptosis detection kit was ordered from BioLegend (San Diego, CA). LDH Assay Kit was purchased from Kiazist (Hamedan, Iran). The reagents used in the cell culture were all obtained from Gibco-Thermo Fisher Scientific, USA.

Breast Cancer Cell Lines and Cell Culture

Human breast cancer cell lines SK-BR-3 and MDA-MB-123 (Iranian Biological Resource Center (IBRC, Iran)) were cultivated in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells were cultured on polystyrene flasks sterilized by gamma irradiation and maintained under 5% CO_2 in a humidified chamber at 37°C.

Drug-to-antibody Ratio (DAR) Quantification

The absorbance of the trastuzumab and DM1 along with the molar absorptivity for each molecule at both 280 nm and 252 nm were used to quantitate the DAR value for the synthesized conjugate. A stock solution of DM1 in dimethyl acetate and of the antibody in reaction buffer containing 5 mM NaCl, 50 mM KH2PO4, and 2 mM ethylenediaminetetraacetic acid (EDTA), pH=7.5 were used to make the standards for plotting the calibration curves. In the next step, the following equation was used for the determination of DAR value, as reported elsewhere (23):

Where A252 nm and A280 nm represent absorbance at 252 nm and 280 nm, $\varepsilon^{DM1}_{252\,nm}$ and $\varepsilon^{DM1}_{280\,nm}$ show molar absorptivities for DM1 at 280 nm and 252 nm, $\varepsilon^{Ab}_{252\,nm}$ and $\varepsilon^{Ab}_{280\,nm}$ represent molar absorptivities for the trastuzumab at 280 nm and 252 nm.

Measurement of free DM1 Content

Free drug content was determined using high-performance reverse phase liquid chromatography (RP-HPLC) (GE Healthcare, Wilmington, MA) (24). Prior to HPLC analysis, the free DM1 was extracted from

$$DAR = \frac{[DM1]}{[Ab]} = \frac{\{A252\ nm - (A280\ nm \times \frac{\varepsilon^{Ab}_{252\,nm}}{\varepsilon^{Ab}_{280\,nm}})\}/\{\varepsilon^{DM1}_{252\,nm} - (\varepsilon^{DM1}_{280\,nm} \times \frac{\varepsilon^{Ab}_{252\,nm}}{\varepsilon^{Ab}_{280\,nm}})\}}{\{A280\ nm - (A252\ nm \times \frac{\varepsilon^{DM1}_{280\,nm}}{\varepsilon^{DM1}_{252\,nm}})\}/\{\varepsilon^{Ab}_{252\,nm} - (\varepsilon^{Ab}_{252\,nm} \times \frac{\varepsilon^{DM1}_{280\,nm}}{\varepsilon^{DM1}_{252\,nm}})\}}$$

the T-DM1 and the trastuzumab-MCC-DM1 samples using a methanol precipitation step. In brief, 2.5 volumes of methanol were added to the samples, and following 20 min of centrifugation at 14,000 rpm, the supernatant (15 µl) was injected into the HPLC system for analyzing the presence of unconjugated DM1 drug species. Identification of peaks corresponding to free DM1 was done with a UV/VIS detector at λmax=252. Dilution series of DM1 standards were prepared with an acetonitrile-based diluent, and then a calibration graph was created by plotting the peak areas versus concentrations of the injected DM1 standards. Finally, the relative percentage of unconjugated DM1 to the trastuzumab-bound drug in the ADC sample was obtained using the following equation (24): mol/mol (%)=[(free drug concentration)/(ADC concentration×DAR)]×100

SDS-PAGE Analysis

The difference in molecular weights of the unconjugated trastuzumab, trastuzumab-MCC-DM1, and T-DM1 was analyzed on a 16% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The protein bands were then visualized with Coomassie brilliant blue staining.

HER2-binding Affinity Assay

Binding efficiency of the trastuzumab after DM1 conjugation was evaluated with an in-cell enzyme-linked immunosorbent assay (ELISA). SK-BR-3 and MDA-MB-231 breast cancer cell lines were plated at a density of 1×10^4 cells/well in 100 µl culture medium in 96-well plates and incubated at 37°C under 5% CO_2 for a period of 24 h. The cells, subsequently, were fixed for 30 min at room temperature with paraformaldehyde in phosphate-buffered saline (PBS) (4% v/v), then the culture medium was discarded. Following three rinses with PBS, the excess non-specific protein-binding sites were blocked with bovine serum albumin (BSA)/PBS (3% w/v) for 1-2 h at room temperature with mild shaking. Next, the blocking buffer was aspirated off, and the cells were incubated with increasing concentrations of trastuzumab, trastuzumab-MCC-DM1, T-DM1, and BSA for 1 h at room temperature with gentle agitation. Then three-fold washing with 0.05% Tween 20/PBS was performed and the cells were incubated with 100 µl of goat anti-human IgG-horseradish peroxidase (HRP) (dilution 1: 10,000 in 0.1% BSA/PBS) for 1 h at room temperature. The plate was rinsed three times with 0.05% Tween 20/PBS, and then substrate solution (3,3',5,5'-tetramethylbenzidine substrate, 0.1 mg/ml, 100 µl) (Sigma-Aldrich, MO, USA) was added. After 30 min of color development, 50 µl of 1M H_2SO_4 was added to each well to quench the reaction, and the absorbance at λmax=450 nm was thereafter recorded using an ELISA plate reader (Bio-Rad, CA, USA).

MTT Cell Viability Assay

The growth inhibitory activity on MDA-MB-231 and SK-BR-3 cell lines with different HER2-expression profiles was evaluated by the colorimetric MTT viability assay (25). In brief, 1×10^4 cells/well were plated in 96-well plates, and after 24 h were exposed to the trastuzumab, trastuzumab-MCC-DM1, T-DM1, and unconjugated DM1 (with a dose equivalent to the payload content in the trastuzumab-MCC-DM1 ADC) at final concentrations in the range of 0.001-10 µg/ml of antibody/ADC or µM concentration equivalents of bound DM1 (DM1 concentration (µM)=ADC concentration (µg/ml)×DAR/ADC molecular weight (g/mol)). Following an incubation period of 72 h, the rinsing of the cells was performed twice with PBS, and MTT reagent (20 µl, 5 mg/ml in PBS) was then added to each well. After another 3 h incubation at 37°C, insoluble crystals of formazan were solubilized in 100 µl of DMSO. The samples were left for a further 5-10 min at 37°C and the absorbance was recorded using a microplate reader (Bio-Rad, CA, USA) at λmax=570 nm. Experiments were carried out in triplicate, and the cell

cultures without antibody supplementation were included as the controls. Finally, the relative viability of the treated cells in comparison with the untreated control was calculated as follows: %viability=([OD] the test/[OD] the control)×100, where the test and the control represent cell culture pretreated with or without test compounds, respectively.

LDH Release Measurement

The cell toxicity induced by the trastuzumab, trastuzumab-MCC-DM1, and T-DM1 was evaluated by measuring the activity of LDH released by the MDA-MB-231 and SK-BR-3 cells into the culture medium. In brief, the cells were incubated for an exposure time of 72 h with each test compound at a concentration of 1 µg/ml. At the end of the treatments, a working solution containing LDH assay and co-substrate buffers was added to the culture supernatants. After 30 min, LDH release from the dead/damaged cells was quantitated for triplicate samples by measuring the absorbance at λmax=570 nm. Maximum LDH release was determined by adding PermiSolution into the wells and served as the high control. Two other controls were also assayed in parallel with the test compound-treated cells, including the background along with the low controls. The percent of the cytotoxicity was calculated using the following equation: (test sample – low control) / (high control – low control).

Apoptosis Assay

The percentage apoptosis of SK-BR-3 and MDA-MB-231 cell lines in response to the antitumor effect of the test compounds was assayed using an Annexin V-FITC/7-AAD apoptosis kit. Experiments were done according to the kit supplier's recommendation. In brief, the cells were treated with the trastuzumab-MCC-DM1, trastuzumab, and T-DM1 (1 µg/ml) at 1×10^4 cells/well in a 96-well plate and incubated for 48 h. Similarly, the non-treated cancer cells were cultured as the controls for comparison. After being washed with PBS-FBS (4%), the cells were resuspended in Annexin V Binding Buffer at a final concentration of 1×10^6/ml. For cell double-labeling, a total of 5 µl of FITC-Annexin V and 5 µl of 7-AAD viability staining solution were added to 100 µl of the cell suspension, and vortexed gently before incubation at room temperature for 15 min in the dark. Finally, 400 µl of Annexin V binding buffer was added to the samples, and the data were then collected on a FACSCalibur™ Flow Cytometer instrument (BD bioscience, CA, USA) in green and red (FL1 and FL3) cytometric channels.

Statistical Analysis

GraphPad Prism (version 8.0; GraphPad Software, Inc., San Diego, CA, USA) software was used to analyze the data from the in vitro drug activity assays. Using analysis of variance (ANOVA) followed by Tukey's posttest for multiple comparisons, the antiproliferative and antitumor activity of the three drugs were evaluated by comparing the means of live/dead cells after treatments. The triplicate test samples were examined, and the statistical significance was determined by *p*-values less than 0.05.

RESULTS

Molecular Weight Characterization of Trastuzumab-MCC-DM1 Conjugate

The synthesized trastuzumab-MCC-DM1 conjugate, trastuzumab, and T-DM1 were subjected to 16% SDS-PAGE analysis under reducing conditions (Fig. 1). All three species appeared as two individual bands on the SDS-PAGE gel, one at 50-51 kDa and the other at 25-26 kDa, corresponding to the antibodies' heavy and light chains in their unconjugated and conjugated states, respectively. The UV-VIS absorbance spectra of the DM1 drug, trastuzumab, and the trastuzumab-MCC-DM1 also confirmed an increase in absorbance at 252 nm in our synthesized conjugate, signifying the binding of DM1 molecules to the antibody (data not included).

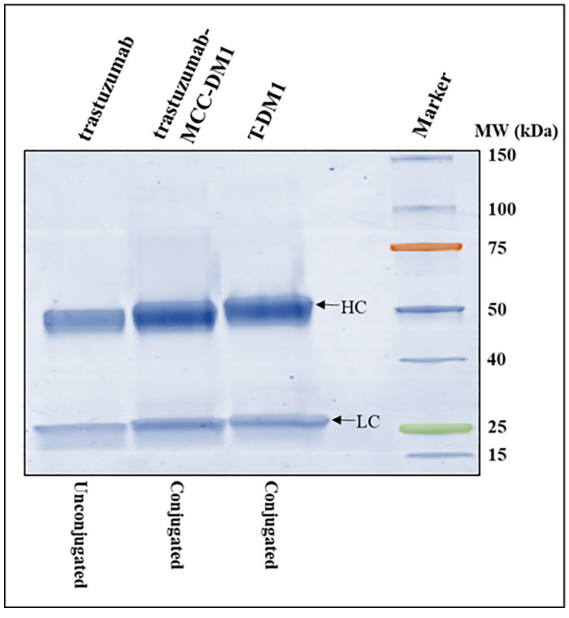

Fig. 1. SDS-PAGE analysis of three different HER2-targeting ADC formats including trastuzumab, trastuzumab-MCC-DM1, and T-DM1 performed under reducing conditions. On the gel, banding patterns associated with trastuzumab, trastuzumab-MCC-DM1, and protein molecular weight marker are depicted. HC and LC: Single heavy and light chains of the antibodies appeared as the upper and lower bands with sizes of 50 kDa and 25 kDa, respectively, in the unconjugated state. At the same time, the conjugated state of heavy and light chains demonstrates an increase of about 1 kDa in size, attributed to the linker-drug complex. MCC: 4-(N-maleimidomethyl) cyclohexane-1-carboxylate; T-DM1: Trastuzumab emtansine, trastuzumab-DM1; HC: heavy chain; LC: light chain

Analysis of the DAR for the Trastuzumab-MCC-DM1 Conjugate

An assessment of the mean loading of DM1 on the trastuzumab was conducted via UV-VIS spectroscopy analysis, and the calculations made based on the molar absorptivity and light absorbance of the two molecules at 252 and 280 nm. Our results showed that on average, 2.9 DM1 drug molecules per antibody were integrated into each trastuzumab-MCC-DM1 conjugate molecule.

Determination of the Free DM1 drug in the Trastuzumab-MCC-DM1 Conjugate Sample

Quantification of free DM1 drug in conjugate solution was conducted by RP-HPLC analysis and the DM1 drug standard curve, depicted in Fig. 2. The level of free DM1 was 2.5%.

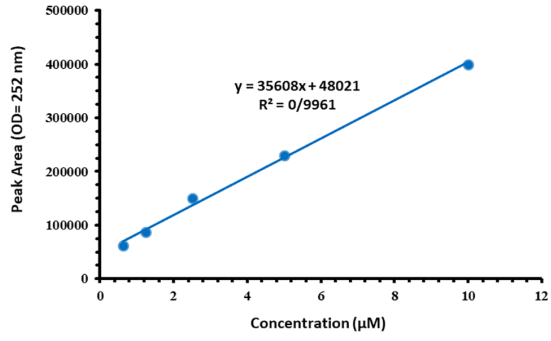

Fig. 2. The calibration graph used to determine the amount of residual DM1 drug in the trastuzumab-MCC-DM1 and T-DM1 samples. A set of dilutions were prepared from the DM1 stock solution, each dilution was injected three times into the column, and the corresponding chromatograms were recorded. The average area under the peak for each dilution was calculated using these chromatograms. The calibration curve was constructed by plotting the concentration of the DM1 against the mean peak area. The regression equation of this curve was computed and later used to estimate the amount of DM1 in the ADC solution.

Evaluation of in vitro Cytotoxicity of Trastuzumab-MCC-DM1 Conjugate

Effects of Drug Conjugation on the Trastuzumab Binding Properties

The binding affinity of the trastuzumab-MCC-DM1 was compared with the unmodified trastuzumab by in-cell ELISA. We examined whether drug coupling had any effect on the antigen-binding efficiency of trastuzumab using the breast cancer cell lines SK-BR-3 (with HER2 positivity) and MDA-MB-231 (with HER2 negativity). As depicted in Fig. 3, we demonstrated that the trastuzumab-MCC-DM1 was capable of binding its target HER-2 antigen on the surface of the SK-BR-3 cell line as efficiently as the unconjugated antibody. Conversely, no significant binding to the MDA-MB-231 cells was recorded for either the trastuzumab or the ADCs.

The Effect of the Trastuzumab-MCC-DM1 Conjugate on the Viability of Cancer Cell Lines

Using the MTT cell viability assay, the

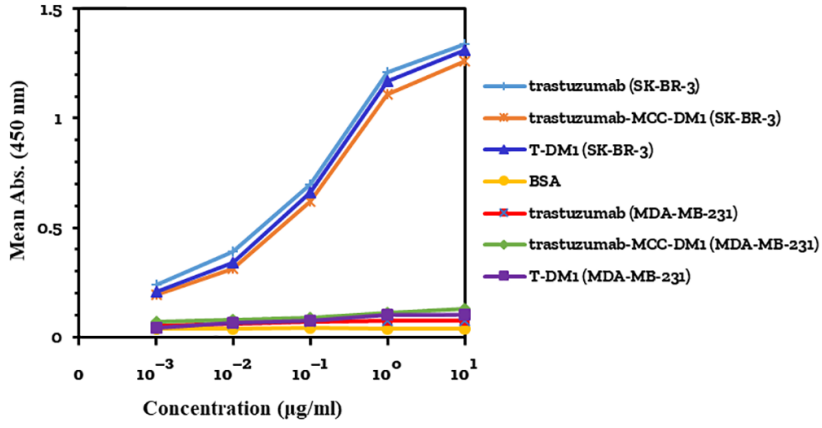

Fig. 3. The in vitro HER2-binding of the unconjugated trastuzumab, trastuzumab-MCC-DM1, and T-DM1 were examined by in-cell ELISA. The three test compounds bound to SK-BR-3 cells (HER2 positive) with similar binding affinities but not to the MDA-MB-231 cells (HER2 negative). Data values are reported as the mean OD data at 450 nm recorded from three independent experiments for ADCs compared with the unconjugated trastuzumab. MCC: 4-(N-maleimidomethyl) cyclohexane-1-carboxylate; T-DM1: Trastuzumab emtansine, trastuzumab-DM1

cytotoxic and antitumor potential of the trastuzumab-MCC-DM1 conjugate against target SK-BR-3 and MDA-MB-231 cell lines were analyzed and compared with those of the unconjugated trastuzumab and the T-DM1. Our results showed that a significantly enhanced inhibitory effect against SK-BR-3 cells was induced by the trastuzumab-MCC-DM1 and the T-DM1 rather than by the trastuzumab alone. The DM1 conjugation to the antibody conferred a higher antitumor activity to the trastuzumab-MCC-DM1 compared with the unconjugated trastuzumab and the DM1 payload, seen in Fig. 4a. Expectedly, in experiments using the MDA-MB-231 cells, none of these

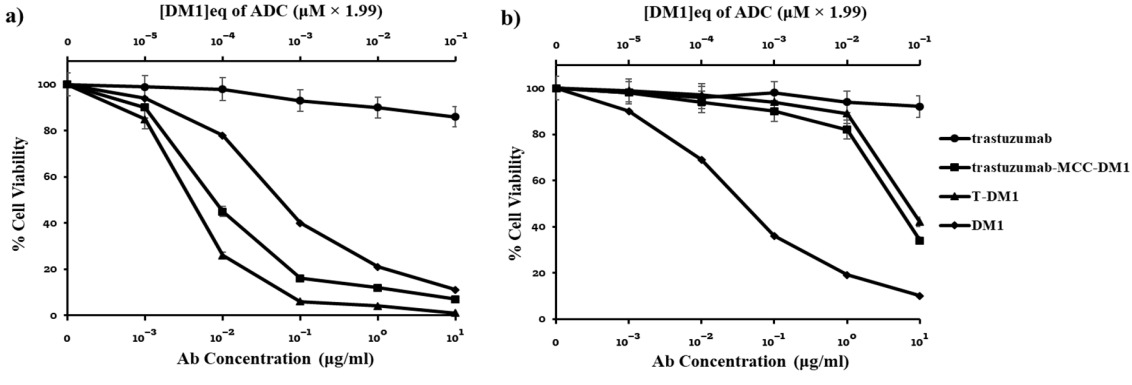

Fig. 4. The growth inhibitory effect of the trastuzumab, trastuzumab-MCC-DM1, T-DM1, and the DM1 payload evaluated on SK-BR-3 (a) and MDA-MB-231 (b) cancer cell lines. The relative viability of these cells was assessed by the MTT assay after a 72-h incubation period. The Trastuzumab-MCC-DM1 and the T-DM1 had a remarkably enhanced growth inhibitory activity compared with the trastuzumab at 0.01 µg/ml ($p<0.05$). Conversely, the trastuzumab was apparently unable to suppress SK-BR-3 cells' survival unless at concentrations exceeding 0.1 µg/ml. Importantly, no difference in proliferation impediment of the MDA-MB-231 cells was observed among trastuzumab, trastuzumab-MCC-DM1, and the T-DM1 at concentrations lower than 1 µg/ml, whereas by increasing the concentrations from 1 to 10 µg/ml ($p<0.05$), reduction in viable cell population occurred with the trastuzumab-MCC-DM1 and the T-DM1. It is likely that these compounds cause off-target cell cytotoxicity at high concentrations. In addition, no significant difference in terms of free DM1 payload cytotoxicity was perceptible between the two cell lines with and without HER-2 positivity. The data values are reported as the mean of percentage inhibition of cell proliferation compared with the control cells obtained from three independent experiments. The error bars represent the standard deviations. ADC: Antibody-drug conjugate; T-DM1: Trastuzumab emtansine, trastuzumab-DM1; MCC: 4-(N-maleimidomethyl) cyclohexane-1-carboxylate

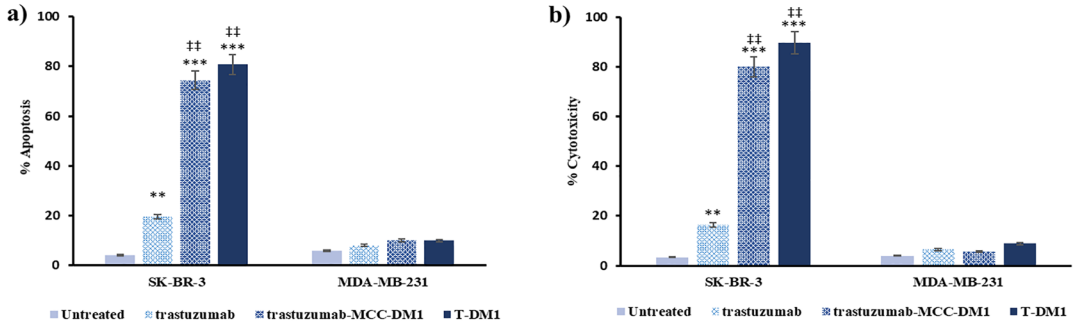

Fig. 5. Cytotoxic and antitumor activity of the immunotherapeutic agent in terms of the percentage apoptosis / LDH release, as a function of the type of cancer cell line (SK-BR-3 and MDA-MB-231) and the HER2-targeting ADC format (trastuzumab-MCC-DM1, T-DM1, and trastuzumab) analyzed with ANOVA in GraphPad Prism 8.0. a) The percentage of apoptotic cancer cells was obtained by treating the cancer cell cultures with these immunotherapeutic agents after a 48-h incubation period. The corresponding untreated cancer cells served as the controls. The cells were stained with Annexin V-FITC/ADD, and analyzed by flow cytometry. b) Lactate dehydrogenase activity measurements were performed using the trastuzumab-MCC-DM1, T-DM1, and the trastuzumab-treated cell cultures and the non-treated controls. A significant increase in the number of apoptotic cells was observed in the cell cultures treated with the trastuzumab-MCC-DM1 and the T-DM1. Similarly, treatment with the three immunotherapeutic agents above indicated a significantly higher level of LDH enzyme activity in SK-BR-3 cancer cell cultures compared with the untreated ones. Importantly, these compounds were unable to induce apoptosis or mediate cell death/damage responsible for LDH enzyme release in the MDA-MB-231 cell line culture medium. The p-values are from two-way ANOVA, followed by Tukey's posttest for multiple comparisons. **p<0.01 and ***p<0.001 are considered significant compared with the respective untreated control. ‡‡p<0.01 compared with the unconjugated species. MCC: 4-(N-maleimidomethyl) cyclohexane-1-carboxylate; FITC: fluorescein isothiocyanate; ADD: Aminoactinomycin D. T-DM1: Trastuzumab emtansine, trastuzumab-DM1

cytotoxic antitumor compounds exerted an inhibitory effect on the viability of the cells at concentrations just below 10 µg/ml (Fig. 4b). Nevertheless, at higher concentrations, the trastuzumab-MCC-DM1 and the T-DM1 exhibited minimal antiproliferative action against the MDA-MB-231 cancer cell line, while the unconjugated DM1 payload had a potency comparable with that on the HER2-overexpressing SK-BR-3. Of note, no growth inhibitory effect against the MDA-MB-231 cells was ascertained when treated with the trastuzumab at 10 µg/ml.

The Proapoptotic Potential of the Trastuzumab-MCC-DM1 Conjugate

The efficacy of the trastuzumab-MCC-DM1 to induce apoptosis through its antitumor activity was evaluated utilizing flow cytometry analysis of breast cancer cell lines with and without HER2 positivity. According to our findings, the trastuzumab-MCC-DM1 and the T-DM1 proved more potent than the trastuzumab at stimulating a strong apoptosis response responsible for a high death rate in SK-BR-3 cells. However, expectedly, the MDA-MB-231 cell line was found not to be susceptible to death following treatment with the unconjugated trastuzumab, the trastuzumab-MCC-DM1, and T-DM1 (Fig. 5a).

LDH Assay for Cytotoxicity Testing

The colorimetric LDH assay was employed to evaluate the cytotoxic potential of the trastuzumab-MCC-DM1 compared with the trastuzumab and the T-DM1. The measurements at 490 nm after 72 h revealed that the trastuzumab-MCC-DM1 and the T-DM1 treatments resulted in substantial LDH release, suggesting that these two test compounds triggered high levels of cytotoxicity towards the SK-BR-3 cell line, shown in Fig. 5b. On the other hand, the supernatant of the trastuzumab-treated SK-BR-3 cell cultures displayed a lower LDH release, indicating a less potent inhibitory effect of the trastuzumab on this cell line. None of the three compounds caused

cytotoxicity in MDA-MB-231 cells, since this cell line is negative for HER2.

DISCUSSION

Biological therapy as an ever-expanding subject of exploration for multiple types of carcinomas has become the focal point of ongoing research. ADCs have been developed as a breakthrough and highly effective therapeutic route for breast cancer treatment, with a superior therapeutic index compared with the preexisting antitumor therapies (17, 26). In an earlier study, we synthesized the trastuzumab-MCC-DM1 conjugate, designed to target cancer cells expressing the HER2 antigen. Conducting the present research, we evaluated the in vitro biological activity and physicochemical properties of this particular ADC compared with those of the unconjugated trastuzumab and T-DM1, which served as the negative and positive controls, respectively, so as to inspect the tumor-suppressive and inhibitory effects of the trastuzumab-MCC-DM1 in the HER2-positive breast cancer subtype.

HER2 is expressed in multiple tissues where it can contribute directly to uncontrolled cell growth and exert its tumorigenic and tumor metastasis-promoting effects (27). Receptor overexpression and/or gene amplification of HER2 has been documented in several malignancies affecting the bladder, ovary, and lung, among others (28-30), and HER2 oncogenic potential has been well documented in preclinical and clinical studies. More importantly, HER2 expression status is considered a predictive marker for tumor responsiveness to HER2-targeted therapies, such as the trastuzumab and lapatinib (31). Cytotoxic payload-coupled antibodies directed against the extracellular domain of HER2 have been exploited in several breast cancer-targeted immunotherapies. ADCs with significantly improved growth inhibitory effects attributed to their DM1 payload, are found to exhibit remarkable outcomes in the trastuzumab-insensitive HER2-expressing cancer cells (17). The initiation of an antitumor response in these cells relies on trastuzumab's recognition and binding to cell-surface HER2, facilitating cellular uptake of ADC. Subsequently, drug payload DM1 can operate through a trastuzumab-independent mechanism of action to exert its effect as an antimitotic agent, thereby contributing to tumor-inhibitory processes inside cancer cells.

The drug-to-antibody ratio (DAR), is a key quality attribute determining the extent of targeted intratumoral delivery of drug payload, thereby directly affecting the ADC safety, efficacy, potency as well as in vitro cytotoxic response stimulated by this therapeutic agent (32). As a key physicochemical characteristic of our synthesized trastuzumab-MCC-DM1, we measured the DAR value for this ADC by UV-VIS spectroscopy analysis. As many as 2.9 DM1 payloads were bound to each trastuzumab in the applied conjugation reaction process, comparable with those of the previous studies involving the ADC synthesis (33). Despite the lower DAR value obtained for trastuzumab-MCC-DM1 compared with the T-DM1, with a DAR of 3.5 (19), the cytotoxicity induced by the two ADCs was not significantly different ($p>0.05$). Supporting these findings, it is noted that, irrespective of the type of cytotoxic substances; antibodies, or linkers incorporated, a majority of conjugates under development in clinical trials contain 2 to 4 cytotoxic drug payloads attached to each antibody. More importantly, the ADCs with a DAR of 4 were shown to be as effective as those with an 8 DAR value in terms of their in vivo antitumor activities at the same antibody levels (34).

ADCs' development can be complicated by probable partial conjugation reactions that may leave behind by-products in the form of unbound free drug molecules, linker-drug complexes, or other drug-derived substances, thereby compromising the purity and therapeutic index of the resulting ADC (35, 36). Given the potentially toxic nature of

maytansinoids, it is of paramount importance that the free drug level in the conjugation reaction end product is appropriately determined while developing DM1-based ADCs. In our analysis, residual non-conjugated DM1 species at a concentration of 2.5% were detected in the ADC samples using RP-HPLC analysis after the removal of the protein fraction from the samples. These results were similar to those of the previous studies (37). The molecular weight was determined by SDS-PAGE as another physicochemical characteristic of the synthesized conjugate in our study. The unconjugated trastuzumab heavy and light chains displayed molecular weights of approximately 50 kDa and 25 kDa, while drug conjugation led to an apparent mass increase of about 1 kDa (MCC-DM1's molecular weight) for both the heavy and light chains. Previous research has reported the SDS-PAGE analysis with similar results in this regard (38). In addition to confirming the trastuzumab-MCC-DM1 structural integrity by SDS-PAGE, the in vitro binding assay using ELISA also revealed the ADC retained a high affinity towards cancer cell lines expressing HER2. We observed a comparable binding efficiency of the trastuzumab-MCC-DM1 in comparison with both the T-DM1 and the unmodified antibody trastuzumab, supporting the fact that the conjugation chemistry did not prejudice the trastuzumab-MCC-DM1's physiochemical qualities, thus not affecting its ability to bind HER-2 antigen. Superior efficacy and safety are regarded as vital attributes of an ADC. In this context, in vitro bioactivity assays were conducted on breast cancer cell lines to explore the cytotoxic, antiproliferative, and proapoptotic effects of the trastuzumab-MCC-DM1 conjugate. Comparing the three test compounds in terms of their efficacy, evaluated by the MTT cell viability assay, a significantly high dose-dependent death response was observed in SK-BR-3 cells treated with the drug-conjugated trastuzumab species. In determining potency as a molar equivalent of the DM1, against SK-BR-3 cells, the DM1 integrated with the trastuzumab proved more potent than the unconjugated DM1 payload. Both SK-BR-3 and MDA-MB-231 cells underwent treatment with the cell-permeable DM1 alone, demonstrating diminished cell survival. At concentrations used in the assay, these three agents failed to induce toxicity towards the MDA-MB-231 cells, as a negative control, due to the lack of HER2 target antigen on the cell surface. The reduction in the MDA-MB-231 cell viability seen with the trastuzumab-MCC-DM1 and T-DM1 at 10 μg/ml suggests that these ADCs may induce nonspecific off-target cytotoxicity at higher concentrations, predominantly due to an increased DM1 payload release. There are reports on off-target toxic effects associated with the T-DM1 conjugates, assumed to be the consequence of either the ADCs or their released free payload being internalized into nontarget cells.

Although the unconjugated trastuzumab has growth-inhibitory effects, it proved ineffective to trigger a significant cytotoxic response in HER2-positive cell lines at concentrations used for cell culture treatment. These data were consistent with the previous studies in which selective ADCs were used to target tumor-specific receptors for the cytotoxicity (39-41). Moreover, the proapoptotic effect of the trastuzumab-MCC-DM1 was found to be similar to that of the T-DM1, while the trastuzumab exhibited a significantly weaker apoptosis-inducing activity against high HER2-expressing SK-BR-3 cells. Conversely, the trastuzumab and its corresponding conjugates did not stimulate an apoptotic response in the HER2-negative MDA-MB-231 cell line. Quantitative measurement of LDH release into the culture medium was carried out to further verify the potent cytotoxic activity of the trastuzumab-MCC-DM1 conjugate against cancer cell lines. The produced formazan crystal level correlates directly with the levels of LDH, which is in turn directly proportional to the number of dead or membrane-ruptured

cells in the culture. The percentage of LDH activity was found to be the lowest for the trastuzumab at similar levels as its corresponding conjugates, and no significant difference was observed between the two drug-conjugated trastuzumab, suggesting that the trastuzumab maintains its ability to induce cell death response upon conjugating with DM1. These results were consistent with the previous studies (41). Statistically, there was no significant difference in LDH release from HER2-negative cancer cells exposed either to trastuzumab-MCC-DM1, T-DM1, or the trastuzumab. In this way, our study further showed that conjugating the trastuzumab with DM1 improved its antitumor and antiproliferative effects in vitro, as stated in a previous study (42).

CONCLUSION

In the current study, a previously developed HER2-targeted cancer immunoconjugate called trastuzumab-MCC-DM1 was characterized. The data obtained from evaluations on LDH release, cancer cell apoptosis, and cell viability illustrated the synthesized trastuzumab-MCC-DM1 was able to exhibit significantly improved in vitro tumor-suppressive effects in HER2-positive breast cancer cells compared with the trastuzumab alone. These findings verify that stimulating proapoptotic and antiproliferative responses in cancer cells is associated mainly with DM1 drug payload. This ADC, however, may exert off-target antigen-independent cytotoxic effects at higher concentrations. Therefore, these in vitro findings should be extended to in vivo settings to further evaluate the trastuzumab-MCC-DM1 for efficacy and safety on an ongoing basis.

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CONFLICT OF INTEREST

None declared.

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