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Immunological Responses against HER2-targeted Idarubicin-Z_{HER2} Conjugate in BALB/c Mice

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ABSTRACT

Targeting of cancerous cells with a high level of human epidermal growth factor receptor 2 (HER2) expressions by drug immunoconjugates is a new approach for specific delivery of chemotherapeutic agents. Our previous work indicated that idarubicin-Z_{HER2} affibody conjugate has a great potential for the treatment of HER2-overexpressing malignant cell lines but possible induced immune response against constructed conjugate was not addressed.

In the current study, the possibility of induction of humoral and cellular immune responses against idarubicin-Z_{HER2} affibody conjugate in BALB/c mice was investigated. For assessment of the induced immune response, prepared and qualified idarubicin-Z_{HER2} affibody conjugate was administrated intravenously to BALB/c mice and the induced cellular immune response was evaluated by measuring secretion levels of interferon gamma (IFN-γ) and interleukin 10 (IL-10) cytokines by the splenocytes. Humoral response of treated mice was also assessed by measuring total immunoglobulin G (IgG) titer in mice sera.

The obtained results showed that idarubicin-Z_{HER2} affibody conjugate at any examined concentrations could not induce secretion of IFN-γ as a pro-inflammatory cytokine. A mild increase in the level of regulatory IL-10 cytokine was seen in the treated mice although no dose dependency in the level of IL-10 production was observed. Furthermore, results showed that idarubicin-Z_{HER2} conjugate could not induce IgG production in the treated mice.

Based on these findings, the idarubicin-Z_{HER2} conjugate can be considered as a candidate for the development of new therapeutics against HER2-overexpressing cancers although further *in vivo* studies are needed.

Keywords: Cytokine; Human epidermal growth factor receptor 2; Idarubicin-Z_{HER2} affibody conjugate; Immune response; Immunoglobulin G

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INTRODUCTION

Members of the human epidermal growth factor receptor (HER) family have vital roles in the normal growth, survival, adhesion, immigration, and differentiation of various kinds of mammalian cells.[1] This family contains four closely related types of transmembrane tyrosine kinase receptors; HER1, HER2, HER3, and HER4 which also known as ErbB1, ErbB2, ErbB3, and ErbB4, respectively.[2] Each HER molecule composed of an extracellular domain for binding of related ligands, a transmembrane lipophilic segment and an intracellular tyrosine kinase domain.[2] Dimerization of HER receptors in the presence of related ligands is an essential step for their activation and initiation of signal transduction.[2,3] As an exception, HER2 can fold into the active structure in the absence of a known ligand.[4] The HER2 abnormal expression and signaling have been associated with the development and progression of several kinds of malignancies including human breast cancer,[5] ovarian cancer,[6] gastric carcinoma,[7] and salivary gland tumors.[8] Over-expression of HER2 is also associated with enhanced invasiveness of cancer cells and their resistance to chemotherapy or radiotherapy.[9,10] Thus, over-expression and unusual ligand-independent activation of HER2 could have resulted in the development of various kinds of malignancies in various tissues.[11-13]

Monoclonal antibodies such as trastuzumab (Herceptin) are currently used for the treatment of malignancies with over-expression of HER2. This kind of humanized antibody binds to the extracellular domain of the HER2 and consequently inhibits its dimerization and activation.[5] On the other hand, small-molecule inhibitors such as lapatinib and neratinib inhibit the enzymatic tyrosine kinase activity of HER2 and block its downstream receptor signaling pathway.[14] Antibody-drug conjugates (ADCs) like emtansine (Trastuzumab-DM1) are a new generation of drugs for specific targeting and ablation of HER2-overexpressed malignant cells.[15] These chimeric structures could deliver conjugated drugs to targeted cells more efficiently.[16] Alternatively, the antibody moiety of a drug immunoconjugate can be replaced with an affibody molecule. Affibodies are engineered thermo stable peptides which were originally generated from the B-domain of the immunoglobulin binding region of staphylococcal protein A.[17] Due to some advantages, affibody molecules are a valuable device for targeted drug delivery and tumor imaging.[18] Affibody Z_{HER2} has been used for delivery of various anti-cancer drugs to HER2-positive cancerous cell lines.[19-21] Our previous *in vitro* studies indicated that idarubicin-Z_{HER2} affibody conjugate can be successfully used for specific ablation of HN5 cell line as a HER2-positive head and neck squamous cell carcinoma (HNSCC) and MCF-7 breast cancer cell line.[22] But, in vivo immunological responses to this conjugate is not yet investigated and remained to be examined. Antibody-drug conjugates (ADC) responses may be developed against each of the three main moieties of a drug immunoconjugate including protein, linker or small-molecule drug. The current study aims to investigate the possibility of induction of humoral and cellular immune responses against idarubicin-Z_{HER2} affibody conjugate in BALB/c mice. Our results showed that idarubicin-Z_{HER2} affibody conjugate could not induce secretion of interferon gamma (IFN-γ) as a pro-inflammatory cytokine. A mild but not dose-dependent increase in the level of regulatory interleukin 10 (IL-10) cytokine was seen in the treated mice. Additionally, the obtained results showed that idarubicin-Z_{HER2} conjugate is not capable to induce IgG production in the treated mice.

MATERIALS AND METHODS

Chemicals

Idarubicin was purchased from Selleckchem (Houston, USA). Sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC), HisPur™ Ni-NTA resin, imidazole, and β-D-1- thiogalactopyranoside (IPTG) were obtained from Thermo Fisher Scientific (Massachusetts, United States). Ethylenediaminetetraacetic acid (EDTA), ampicillin, phenylmethanesulfonyl fluoride (PMSF), 3-[4, 5, dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) and RPMI-1640 were purchased from Sigma-Aldrich (St. Louis, MO, USA). BALB/c mice were purchased from the animal house of Jundishapur University of Medical Sciences (IR.AJUMS.REC.1395.406). All other chemicals were obtained from Merck (Darmstadt, Germany). IFN-γ and IL-10 ELISA kits were purchased from BOSTER Co. (CA, USA).

Expression and Purification of Z_{HER2} Affibody

Gene of Z_{HER2} affibody was synthesized by *de novo* gene synthesis according to the available sequence.[23] One cysteine codon was added to its 5´ end. The synthesized Z_{HER2} affibody gene was inserted into a Champion™ pET302/NT-His plasmid from Invitrogen™ (Thermo Fisher Scientific, USA) and cloned in competent *E. coli BL21* cells.[24] Affibody expression was performed as described previously.[22] For affibody purification, the culture medium was centrifuged (2000×g for 15 min) and then the bacterial pellet was collected and sonicated in lysis buffer (25 mM Tris-HCl, 1.0 mM EDTA and 1.0 mM PMSF, pH 6.8). Sonication was performed five times, 30 seconds each, using a probe sonicator (UP50H, Hielscher, Teltow, Germany). The cell lysate was centrifuged (6000×g for 15 min at 4°C) and then, the supernatant was collected for affibody purification. Purification of recombinant Z_{HER2} affibody *via* His-select affinity column chromatography was performed using HisPur™ Ni-NTA resin (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. Column eluted fractions were analyzed by Coomassie brilliant blue staining following sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) to confirm the presence of affibody protein band. Affibody-containing fractions were pooled and concentration of protein was determined using Bradford reagent.[25]

Preparations of Idarubicin-Z_{HER2} Affibody Conjugate

Idarubicin-Z_{HER2} affibody conjugate was prepared as described previously[22] with some modifications. Conjugation was performed using sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC) as a heterobifunctional crosslinker that allows covalent conjugation of amine- and sulfhydryl-containing molecules through the formation of stable thioether bonds. Sulfo-SMCC in DMSO was diluted with conjugation buffer (PBS, pH 7.2 supplemented with EDTA 2.0 mM), and then was added to the idarubicin solution (1.0 mg mL^{-1}) at a 2-fold molar excess. The reaction mixture was incubated for 30 min at room temperature and then, affibody solution was added to the mixture in a 1:1 molar ratio with idarubicin. After 30 min, the unreacted cross-linker was removed using a desalting column equilibrated with conjugation buffer. The conjugated product was concentrated using Amicon Ultra-15 centrifugal filter unit. Construction of idarubicin-Z_{HER2} affibody conjugate was confirmed using UV-Vis Spectroscopy (SPECORD 210 plus, Analytik Jena, Germany) and tryptophan fluorescence spectroscopy (LS-45 Fluorescence spectrometer, Perkin-Elmer, USA).

Animal Treatment

Twenty-one female BALB/c mice (4-6 weeks) were obtained from Research Center and Experimental Animal House, Jundishapur University of Medical Sciences, Ahvaz, Iran. Animal experiments were approved by the Animal Research Ethics Committee of Ahvaz Jundishapur University of Medical Sciences (IR.AJUMS.REC.1395.406). Animal care during the study was per under the NIH Guide for Care and Use of Laboratory Animals (NIH 8th Edition).[26] Mice were acclimatized for one week before the experiment and then, randomly divided into seven groups with three members in each group. The mice were intravenously administered with idarubicin-Z_{HER2} conjugate (0.2-1.2 mg kg^{-1}). The mice in the negative control group were treated by PBS and BCG (Bacillus Calmette-Guérin)-treated mice were used as positive control. The treated mice were boosted three times at two weeks intervals and euthanized two weeks after the last booster. After the sacrifice of the treated mice, their spleens were aseptically removed and suspended in the culture medium individually.

Measurement of Cell Viability

The MTT assay was used to determine the viability of the cultured splenocytes by measuring the mitochondrial function.[27] Splenocytes were seeded into 96-well plates (2×10^5 cells $well^{-1}$) and after overnight incubation; idarubicin-Z_{HER2} affibody conjugate was added at different concentrations (4.4 to 26.4 µg mL^{-1}). After incubation for 24 hours, the supernatant was gently removed and 20 µl of MTT dye solution (5.0 mg mL^{-1} in PBS) was added to each well. The culture plates were incubated for another 4 hours. The formazan blue-colored product was solubilized by addition of DMSO and OD_{570} values were recorded by SpectraMax M5 microplate reader (Molecular Devices, USA).

Evaluation of Induced Cytokine Response to the Idarubicin-Z_{HER2} Immunoconjugate

The spleens were meshed and suspended in RPMI 1640 medium. Red blood cells were lysed using lysis buffer (NH_4Cl 0.16 N, Tris 20 mM, pH 7.2) and the

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cell suspensions were centrifuged at 600×g for 10 min. After washing with RPMI 1640, lymphocyte pellets were re-suspended at 2×10^6 cells mL^{-1}. [28] in complete RPMI 1640 medium and stimulated with idarubicin-Z_{HER2} affibody conjugate at a final concentration of 26.4 µg mL^{-1}. The cultured cells were incubated at 37°C in a humidified incubator with 5% CO_2. The level of IL-10 secreted in the culture medium at 24 hours was measured in the culture supernatant using mouse IL10 ELISA Kit with the detection limit of <1pg mL^{-1} (15.6pg mL^{-1}-1000pg mL^{-1}), the intra-assay and inter-assay precisions were 4.1% and 6%, respectively. Also, the level of IFN-γ secreted in the culture medium at 96 hours was measured in the culture supernatant using a mouse IFN-γ ELISA Kit with the detection limit of < 5 pg mL^{-1} (31.2 pg mL^{-1}-2000pg mL^{-1}), the intra-assay and inter-assay precisions were 4.3% and 4.4%, respectively. The amount of the produced IFN-γ and IL-10 by spleen cells was expressed as pg mL^{-1}.

Statistical Analysis

All experiments were performed in triplicate and repeated three times. Data were analyzed using Student's t-test by statistical software package for Windows, version 15.0 (SPSS, IBM Corp, Armonk, NY, USA) and presented as mean ± SEM. Values of $p<0.05$ were considered as statistically significant.

RESULTS

Preparation and Characterization of Idarubicin-Z_{HER2} Conjugate

Affibody Z_{HER2} with a hexahistidine-tag (6xHis-tag) was cloned and expressed in the bacterial host, *BL21*. Expressed affibody molecules were purified using Ni-affinity column and purification of the product was confirmed by SDS-PAGE analysis of the column effluent. As presented in Figure 1, a distinct protein band with a molecular weight of around 12 kDa is seen in the Coomassie blue-stained gel in accordance with the previous publications.[22,29,30] As described above in the materials and method section, affibody product was covalently bound to idarubicin through heterobifunctional sulfo-SMCC crosslinker. Sulfo-SMCC allows covalent conjugation of amine-containing (idarubicin) and sulfhydryl-containing (Z_{HER2} affibody) molecules. Conjugation was confirmed by characterization of purified idarubicin-Z_{HER2} product using UV-Vis and fluorescence spectroscopy as described previously by us in details.[22]

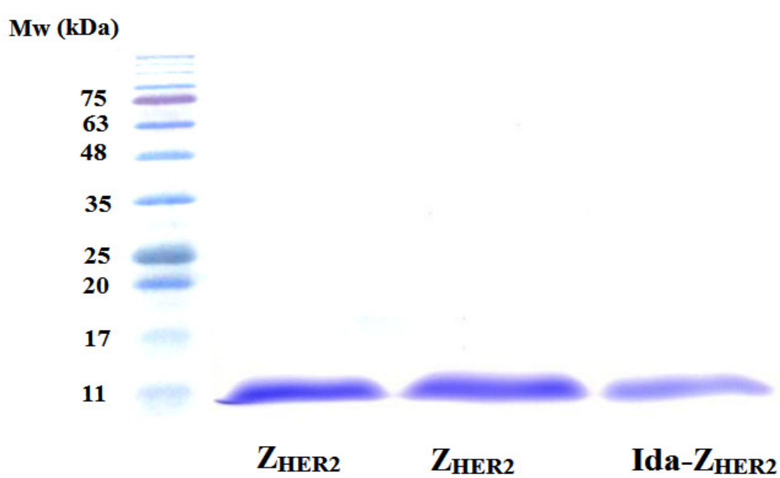

Figure 1. Gel electrophoresis of purified Z_{HER2} affibody molecule and idarubicin-Z_{HER2} affibody conjugate using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis in reducing condition on SDS-PAGE (12% resolving and 5% stacking), the gel was stained with Coomassie brilliant blue. The existence of a single protein band with an apparent molecular weight of 12 kDa confirmed the purity of the Z_{HER2} affibody extracted.

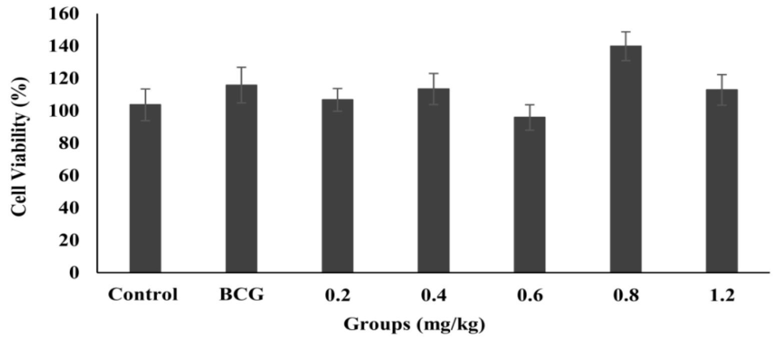

Figure 2. Cytotoxicity assessment of constructed idarubicin-Z_{HER2} affibody. Data are presented as means ± SEM and the mean of each group compared to the control group by independent sample *t-test*. Splenocytes of mice which intravenously administrated with idarubicin- Z_{HER2} affibody were incubated in the presence of various concentrations of idarubicin-Z_{HER2} affibody (4.4 to 26.4 µg mL^{-1}) for 24 hours and then cell viability was assessed using 3- [4, 5, dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT).

Toxicity of Idarubicin-Z_{HER2} Conjugate against Spleen Cells

The results of the MTT assay for evaluation of idarubicin-Z_{HER2} conjugate cytotoxicity against isolated splenocytes are depicted in Figure 2. No significant decrease in cell viability was observed after 24 hours of exposure to examined concentrations of the drug conjugate. Based on the obtained results, the constructed idarubicin-Z_{HER2} conjugate seems to be nontoxic at *in vitro* conditions in the examined concentration range.

Induction of IL-10 and IFN-γ Production by Idarubicin-Z_{HER2} Affibody Conjugate

As shown in Figure 3, IFN-γ as a pro-inflammatory cytokine is induced (mean value of 2570 pg mL^{-1}) by BCG, while, idarubicin-Z_{HER2} conjugate at any examined dose could not induce IFN-γ response in treated mice. The expression level of IFN-γ in mice intravenously administrated with conjugate (mean value of 20-30 pg mL^{-1}) was in the order of its expression in the control untreated mice (mean value of 20 pg mL^{-1}).

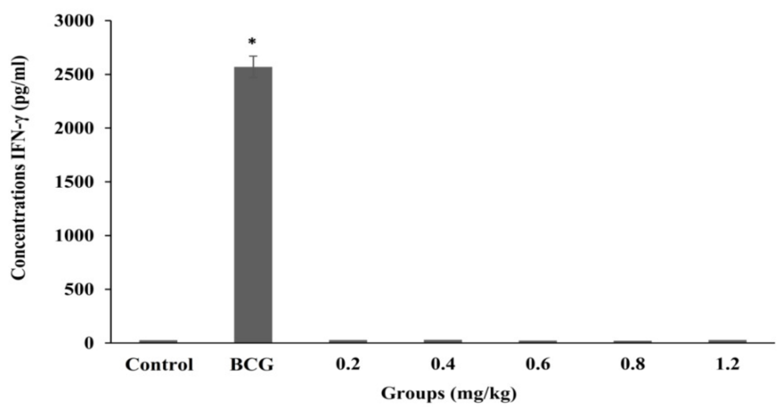

Figure 3. Assessment of interferon- gamma (IFN-γ) production by splenocytes derived from mice treated with idarubicin-Z_{HER2} affibody conjugate. Data are presented as means ± SEM and the mean of each group compared to the control group by independent sample *t*-test (n=3). Idarubicin-Z_{HER2} affibody conjugate at different doses (0.2-1.2 mg kg^{-1}) was intravenously administrated to BALB/c mice three times and then spleens of mice were aseptically removed and suspended in the culture medium for *in vitro* assessment of IFN-γ production. Splenocytes were resuspended at 2×10^6 cells mL^{-1} in complete RPMI 1640 medium and stimulated with idarubicin-Z_{HER2} affibody conjugate at a final concentration of 26.4 µg mL^{-1}. Secretion of IFN-γ in the culture medium was measured in the culture supernatant using IFN-γ ELISA kit after 96 h. (*p= 0.011, compared to the untreated control group).

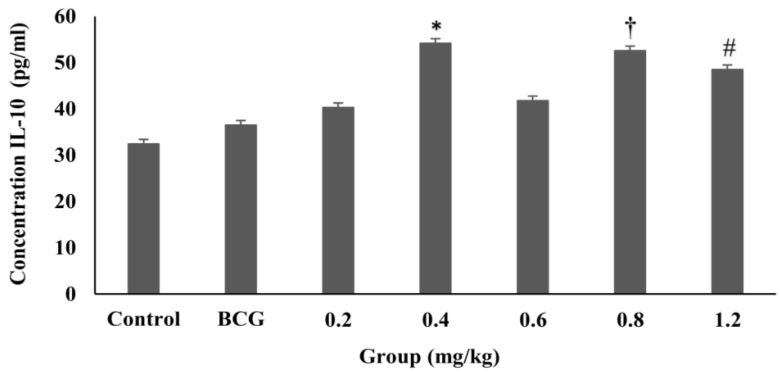

Figure 4. Assessment of interleukin 10 (IL-10) production by splenocytes derived from mice treated with idarubicin-Z_{HER2} affibody conjugate. Data are presented as means ± SEM and the mean of each group compared to the control group by independent sample *t-test* (n=3). Idarubicin-Z_{HER2} affibody conjugate at different doses (0.2-1.2 mg kg^{-1}) was intravenously administrated to BALB/c mice three times and then spleens of mice were aseptically removed and suspended in the culture medium for *in vitro* assessment of IL-10 production. Splenocytes were resuspended at 2×10^6 cells mL^{-1} in complete RPMI 1640 medium and stimulated with idarubicin-Z_{HER2} affibody conjugate at a final concentration of 26.4 µg mL^{-11}. Secretion of IL-10 in the culture medium was measured in the culture supernatant using IL10 ELISA kit after 24 h. (p*=0.005, †*p* = 0.001, $^{\#}p$ = 0.001, compared to the untreated control group).**

A mild increase in the level of regulatory IL-10 cytokine was seen in the mice treated with different concentrations of the idarubicin-Z_{HER2} conjugate (40-54 pg mL^{-1} in the treated mice *vs*. 32 pg mL^{-1} in the untreated control group) (Figure. 4). No dose dependency in the level of IL-10 production was observed after treatment of mice with different concentrations of the idarubicin-Z_{HER2} conjugate. Data presented here showed that idarubicin-Z_{HER2} conjugate cannot induce or suppress the production of cytokines to a significant level.

Humoral Responses of Mice to Idarubicin-Z_{HER2} Affibody Conjugate

In the current study, level of total IgG (IgG titer) in the sera of mice treated with different doses (0.2-1.2 mg kg^{-1}) of idarubicin-Z_{HER2} affibody was measured as an indicator of the induced humoral immune response. Our results (Figure 5) showed that idarubicin-Z_{HER2} conjugate at any examined dose is not capable to induce a significant level of IgG production in the treated mice in comparison to the untreated control group.

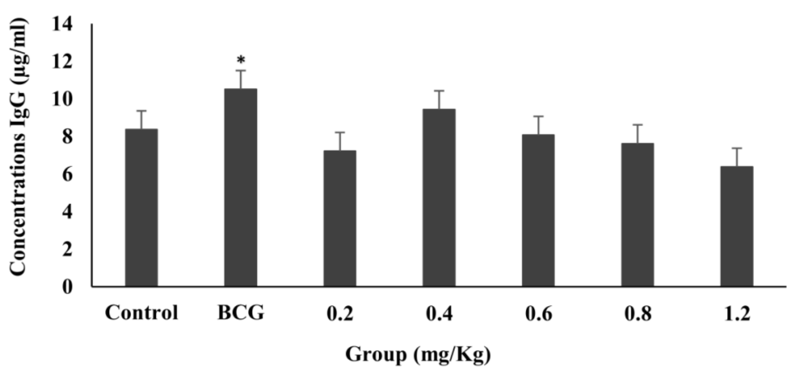

Figure 5. Total immunoglobulin G (IgG) titer in sera of mice treated with Idarubicin-Z_{HER2} affibody conjugate. Data are presented as means±SEM and the mean of each group compared to the control group by independent sample *t-test* (n=3). Idarubicin-Z_{HER2} affibody conjugate at different doses (0.2-1.2 mg kg^{-1}) was intravenously administrated to BALB/c mice three times and then total IgG titer was measured. (p*= 0.001, compared to the untreated control group).**

DISCUSSION

Affibodies, as a new class of engineered affinity proteins, have been investigated for a different application, especially diagnostic imaging and therapy, in the past 20 years. Affibodies due to their improved properties, such as small size, robustness, and high stability has attracted great attention in recent years.[31] Today, several affibody molecules with different specificities have been evaluated for *in vivo* purposes.[32] A key subject in the development of new therapeutic proteins is minimizing their immunogenicity to avoid side effects.[33] To our knowledge, there is no report about the immunotoxicity of affibody-conjugated drugs. So, in the current study, we investigated immune responses elicited against idarubicin-Z_{HER2} conjugate in BALB/c mice.

As the first step, Z_{HER2} affibody was expressed and purified to high purity as shown on SDS-PAGE in Figure 1. The purified recombinant Z_{HER2} affibody showed an apparent molecular weight of 12 kDa which is consistent with the previously published results.[22,29,30] Fluorescence and UV-Vis spectroscopy results confirmed the formation of idarubicin-Z_{HER2} affibody conjugate.[22]

Idarubicin-Z_{HER2} conjugate intended to be used for specific ablation of HER2-overexpressing malignant cells. So, it is crucial to evaluate the toxicity of the prepared drug conjugate against normal cells under *in vitro* and *in vivo* conditions. Spleen as the largest lymphoid organ of mammalians has a critical role in both production/removal of blood cells and also in immunity.[34] Thus, mice splenocytes were employed for cytotoxicity evaluation of conjugated drug using MTT assay. Spleen cells were incubated in the presence of different concentrations of the idarubicin-Z_{HER2} conjugate and cell viability was examined. At *in vitro* condition, our results indicated that the examined concentrations of idarubicin-Z_{HER2} conjugate have no significant effects on the viability of the separated splenocytes.

Drug-polypeptide conjugates may interact with the immune system and enhance/suppress its functions even in the absence of acute cellular toxicity. The immune system may recognize different parts of a drug conjugate as foreign and provoke an immune response which is usually associated with the production of cytokines and antibodies.[35] Currently, the most common approach in testing immunogenicity of bio therapeutics involves measuring antibodies generated against the product but evaluation of immunological responses to bio therapeutic products by measuring the levels of induced cytokines may provide important data for assessment of drug safety and its therapeutic benefits.[36] Cytokines are produced by T cells upon activation and enhance the development of a mature immune response. Among T cells, type 1 T helper (T_H1) cells produce IL-2, IL-3, GM-CSF and IFN- γ and are thought to be involved in the execution of cell-mediated immune response, while type 2 helper (T_H2) cells produce IL-3, IL-4, IL-5, IL-6, and IL-10 and participate in humoral immunity.[37] Production of high levels of cytokines upon exposure to drugs is usually associated with cellular toxicity and unwanted reactions. Therefore, monitoring the levels of pro-inflammatory cytokines and regulatory mediators following administration of polypeptide-drug conjugates can be useful and informative in evaluating therapeutic immune-toxicity or immunogenicity.[38] In the current study, BALB/c mice were injected intravenously (IV) with Z_{HER2}-conjugated idarubicin and then systematic immune response induced by various concentrations of idarubicin-Z_{HER2} was evaluated by measuring secretion levels of two selected cytokines, IL-10 (a regulatory cytokine from T_H2 cells) and IFN-γ (a pro-inflammatory cytokine from T_H1 cells), by splenocytes of treated BALB/c mice at culture condition. BCG-treated mice were used as a control group for the induction of immune response. Mice in the negative control group were treated with PBS solution. Our results indicated that IFN-γ as a pro-inflammatory cytokine is induced by BCG while idarubicin-Z_{HER2} conjugate at any examined dose could not induce IFN-γ response in treated mice compared to control untreated group.

IL-10 inhibits expression of MHC II and co-stimulatory molecule B7-1/B7-2 in monocytes/macrophages which consequently results in the limited production of pro-inflammatory cytokines and chemokines.[39] Additionally, direct action of IL-10 on $CD4^+$ T cells inhibits the production of IFN-γ, interleukin-2 (IL-2), interleukin 4 (IL-4), interleukin 5 (IL5), and tumor necrosis factor -alpha TNF-α Therefore, IL-10 may be mentioned as the suppressor of pro-inflammatory responses in tissues.[40] Our results indicated a mild but not remarkable increase in the level of regulatory IL-10 cytokine and no dose-dependency in the level of IL-10 production was

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observed after treatment of mice with the idarubicin-Z_{HER2} conjugate. Data presented here showed that idarubicin-Z_{HER2} conjugate cannot induce/suppress the production of cytokines to a significant level, a property which is essential for the development of new therapeutics.

It is possible that the conjugation of amino acid sequences to small-molecule drugs through chemical linkers resulted in the generation of novel T cell epitope. $CD4^+$ T cells specific for new antigenic determinants mediate induction of a polyclonal IgG response. In overall, anti-therapeutic antibodies (ATA) may develop against both main moieties of a chemical-peptide conjugate as well as to their linkers.[35] This is a major problem because the developed antibodies may inhibit functions of the conjugated drugs. For non-mucosal routes of drug administration, the expected ATA isotypes are IgM and IgG.[41] Generally, among five antibody isotypes presented in the serum, IgG is the predominant antibody, while, IgA, IgE and IgM levels are very low. Following binding of serum IgG molecules to chemical-peptide conjugate (antigen) and formation of immune complex, complement system may be activated and lead to stimulation of phagocytes to degrade the conjugate.[42]

In the current study, due to some limitations, only the level of total IgG (IgG titer) in the sera of mice treated with different concentrations of idarubicin-Z_{HER2} affibody was measured as an initial screening assay of the induced humoral immune response.[43] Our results indicated that idarubicin-Z_{HER2} conjugate could not induce IgG production in the treated mice in comparison to the untreated ones.

To the best of our knowledge, the current study is the first study to address the effect of the idarubicin-Z_{HER2} conjugate on the induction of immunological responses in BALB/c mice. This interventional study was conducted in a sample of experimental animals BALB/c mice, so, the preclinical outcomes may be expected in further studies. The small sample size and an incomplete panel of the measured Ig isotypes and cytokines are the main limitations of the current study and the results should be interpreted with caution. Further studies with larger sample sizes are required to support these preliminary findings. Finally, by following WHO guideline on biotherapeutic products, it must be stated that immunogenicity should be investigated in the target population after completion of experimental studies in animal and *in vitro* models.

Our previous study indicated that idarubicin-Z_{HER2} affibody conjugate could efficiently decrease the viability of HER2-positive malignant cells compared to HER2-negative cells.[22] Biotherapeutics require a panel of assays to demonstrate a thorough picture of immunogenicity. In the present study, we found that various concentrations of the idarubicin-Z_{HER2} conjugate cannot induce a significant cellular and humoral immune response in BALB/c mice. In conclusion, based on our previous studies and the results of the current investigation, the idarubicin-Z_{HER2} conjugate has great potential as a drug candidate for the specific treatment of patients with HER2-positive tumors, but further *in vitro* and *in vivo* assessments are needed before clinical applications.

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