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Therapeutic Potential of an Anti-PLAC1 Antibody Drug Conjugate in a Murine Model of Human Breast Cancer

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ABSTRACT

Background: *Placenta-specific 1 (PLAC1)* is an oncoplacental gene aberrantly expressed in various malignancies. Antibody-drug conjugates (ADC) offer a promising therapeutic approach by enhancing efficacy and reducing toxicity of treatment compared to cytotoxic small-molecule agents.

Objective: To evaluate the efficacy of an SN38-conjugated monoclonal anti-PLAC1 antibody in a mouse model of breast cancer.

Methods: Anti-human PLAC1 monoclonal antibodies were generated and characterized. SN38 was conjugated to an anti-PLAC1 antibody (clone: 2H12C12) and conjugation efficacy was determined by UV spectrophotometry. The antigen-binding activity of the conjugated antibody was assessed using ELISA and flow cytometry. *In vitro*, the cytotoxic profile of anti-PLAC1-SN38 was evaluated in MDA-MB-231 breast cancer cells using a fluoroimetric viability assay. The impact of anti-PLAC1-SN38 on MDA-MB-231 tumor growth and angiogenesis *ex vivo* was examined using chorioallantoic membrane (CAM) assay followed by immunohistochemical analysis. Pharmacokinetics of anti-PLAC1-SN38 in mice was determined by serial venipuncture following ADC administration. The inhibitory effects of anti-PLAC1 ADC on tumor growth were evaluated in a nude mouse xenograft model of human breast cancer.

Results: The anti-PLAC1 ADC exhibited a substantial cytotoxicity against MDA-MB-231 cells, with effects observed at concentration as low as ~33 nM. In the CAM assay, the ADC significantly reduced the growth of MDA-MB-231 tumor but did not show a significant effect on tumor angiogenesis. Pharmacokinetic analysis in mice demonstrated an average half-life (t1/2) of approximately 80 hours for anti-PLAC1 ADC. In a nude mouse xenograft model, treatment with the ADC resulted in a significant reduction in tumor size compared with isotype-matched antibody-SN38 conjugate, or free SN38.

Conclusion: This study represents the first therapeutic application of anti-PLAC1 ADC in a xenograft model of human breast cancer. Our findings support the embryonic origin of cancers and highlight the potential therapeutic value of targeting oncofetal antigens in human breast cancer.

Keywords: Placenta-specific 1 (PLAC1), SN38, Chorioallantoic membrane, Tumorigenesis, Breast cancer

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INTRODUCTION

Cancer remains a leading cause of death worldwide with breast cancer alone comprising nearly one-third of newly diagnosed malignancies (1). Although surgery, cytotoxic chemotherapy, hormone therapy, and more recently immunotherapy constitute the cornerstone of breast cancer treatments, there remains a pressing need for additional therapeutic strategies.

The recently introduced antibody-drug conjugates (ADC technology) integrate the target specificity of monoclonal antibodies with the cytotoxic potency of chemically linked anti-cancer agents (2). Although extensive research is underway in this area (3), ADC-based therapies have yet to achieve consistently high clinical efficacy . The most critical determinant of any ADC construct in cancer immunotherapy is the molecular target to which the antibody component is directed. Key features of suitable targets include a tumor-specific expression pattern, the presence of an accessible extracellular domain, and efficient internalization upon antibody binding. Additional attributes—such as involvement in essential oncogenic processes like cell proliferation and invasion—may further enhance the therapeutic efficacy of ADCs. Collectively, these characteristics facilitate target selection easier in ADC-based cancer therapies (2).

Oncofetal antigens are proteins typically expressed during fetal development but largely absent in normal adult tissues. Their aberrant re-expression in a range of malignancies, renders them attractive targets for cancer immunotherapy, owing to their limited expression in healthy tissues and strong immunogenic potential. These antigens are frequently implicated in key biological processes such as cell proliferation, migration, and immune evasion, paralleling mechanisms observed in placental development and embryogenesis. Their resurgence in tumors is often associated with poor prognosis, metastasis, and immune suppression, underscoring their therapeutic significance (4). Oncofetal antigens have been utilized in both active and passive immunotherapeutic approaches for cancer treatment (4, 5).

PLAC1 is one of the newly identified oncofetal-placental antigens, predominantly expressed in placental trophoblasts, where it plays a critical role in placental development and function (6-9). The therapeutic appeal of placental antigens lies in their transient expression, restricted to the gestational period. This limited exposure likely circumvents the establishment of central tolerance, thereby preserving their immunogenicity. The evidence supporting the aberrant expression and activation of PLAC1 across a broad spectrum of malignancies is growing (10-12), indicating its potential as a feasibile target for cancer immunotherapy. We recently demonstrated differential expression of PLAC1 in prostate cancer and its positive correlation with Gleason score, underscoring the potential utility of PLAC1 as a target for prostate cancer therapy, particularly in patients with advanced disease (13). Supporting this hypothesis, we subsequently showed that anti-PLAC1-ADC induced apoptosis in human primary prostate cancer cells and prostate cell lines (14), a finding further validated in our subsequent studies involving melanoma cells (15). Moreover, accumulating evidence indicates that PLAC1 is also expressed in breast cancer and related cell lines (16-21). The prevalence of PLAC1 protein expression in breast cancer, as determined by immunohistochemistry (IHC), shows variable results across studies, with reported frequencies typically ranging from 20% to 50%. This variation is influenced by the molecular subtype ofthe tumor and methodological differences in detection protocols (16, 17). While, some studies do not confirm the shedding of PLAC1, others suggest that this protein can be released into circulation and can serve as a potential biomarker for breast cancer (19). Notably, one report describes the binding of secreted PLAC1 to the extracellular matrix, where

it forms a trimeric complex with fibroblast growth factor 7 (FGF7) and its receptor, FGF receptor 2 IIIb (FGFR2IIIb) (22). From a functional perspective, it has been reported that silencing *PLAC1* or targeting it with PLAC1-specific antibodies impairs the motility, migration, and invasion capabilities of breast cancer cells MCF-7 and BT-549 (16). The role of PLAC1 in promoting breast cancer cell invasion and metastasis was further elucidated in a subsequent study (20), which demonstrated that PLAC1 interacted with the proprotein convertase Furin, facilitating the degradation of Notch1 and the generation of Notch1 intracellular domain (NICD) fragments. These NICD fragments were shown to suppress the activity of PTEN (phosphatase and tensin homolog) activity, a key tumor suppressor.

Overall, these findings highlight the potential of PLAC1 as a target for cancer immunotherapy, particularly given its highly restricted expression in normal human tissues (23, 24). Furthermore, based on our observations, PLAC1 is significantly internalized upon engagement with a specific antibody (14). These characteristics support the suitability of PLAC1 as a promising candidate for ADC-based cancer immunotherapy.

Despite their clinical success, current ADCs face significant limitations that compromise both efficacy and safety. A major challenge is on-target, off-tumor toxicity, wherein the target antigen is expressed at low levels in healthy tissues, resulting in dose-limiting damage to normal cells such as those in the skin and eyes. Additionally, tumors often acquire resistance through mechanisms including downregulation of the target antigen, enabling cancer cells to evade therapy, and activation of multidrug resistance efflux pumps that eliminate the cytotoxic payload before it can exert its therapeutic effect. These challenges underscore the urgent need for highly specific, tumor-restricted targets such as PLAC1 (25, 26). Targeting PLAC1 with an ADC may reduce off-tumor toxicity and circumvent resistance mechanisms associated with antigen heterogeneity, offering a more precise and effective therapeutic approach.

While our research team (14, 27, 28) and others (29) have explored the potential of targeting PLAC1 in cancer immunotherapy, there have been no studies to date that have specifically examined PLAC1 targeting for breast cancer immunotherapy. In the present study, we evaluated the therapeutic efficacy of an SN38-conjugated monoclonal anti-PLAC1 antibody using a combination of *in vitro* assays, the chorioallantoic membrane (CAM) assay, and a nude mouse xenograft model of human breast cancer.

MATERIALS AND METHODS

Cell Line and Culture Conditions

The MDA-MB-231 breast cancer cell line was purchased from the National Cell Bank of Iran (Pasteur Institute of Iran, Tehran, Iran) and cultured in DMEM-F12 medium (Gibco, Invitrogen, CA, USA). The culture medium was supplemented with 10% Fetal Bovine Serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin (all from Gibco). Cells were maintained in a humidified incubator at 37˚C with 5% CO2.

Production of Anti-PLAC1 Monoclonal Antibodies

Mouse monoclonal antibodies against PLAC1 were generated as previously described (14), using recombinant human PLAC1 (rhPLAC1) as the immunogen. Based on reactivity with the native protein, assessed via flow cytometry staining of PLAC1-expressing cell lines, clone 2H12C12 was selected. This antibody is specific to human PLAC1 and does not cross-react with mouse plac1. The antibody was purified from mouse ascites fluid using protein G affinity chromatography (Amersham Biosciences, The Netherlands) and characterized through a panel of assays, including enzyme-linked immunosorbent assay (ELISA) (10) Western

blotting (30), and flow cytometry (30).

Preparation of SN38-conjugated Anti-PLAC1 Monoclonal Antibody

The monoclonal anti-PLAC1 antibody was conjugated to SN38 as described in previous studies (14, 15, 31), with minor modifications. The antibody was oxidized using 20 mM sodium periodate for 20 minutes followed by dialysis against 100 mM NaCl and 3 mM acetate buffer (pH 5). The pH of the oxidized antibody was then adjusted to 8 using 15 mM bicarbonate buffer, after which aminated SN38 was added at a mass ratio of 1:3.3 and incubated with shaking for 3 hours. The resulting imine linkage was stabilized with 100 mM sodium cyanoborohydride for 1 hour. Residual unreacted aldehyde groups were blocked using 100 mM ethanolamine for 15 minutes, followed by extensive overnight dialysis against PBS at 4°C. Conjugation efficiency and drug-antibody ratio (DAR) were assessed by ultraviolet–visible spectroscopy and high-performance liquid chromatography (HPLC) as previously reported (14) using a C18 reversed-phase column. The reactivity of anti-PLAC1-SN38 with the immunogen was compared to that of the unconjugated anti-PLAC1 by ELISA. For this assay, a polystyrene 96-well plate was coated with 50 µl of 10µg/ml rhPLAC1 overnight at 4°C. After washing with PBS containing 0.05% Tween 20 (PBS-T), wells were blocked with 5% BSA in PBS at 37 °C for 2 hours and washed again. Wells were then incubated with titrated concentrations (0.7-50 ng/ml) of either anti-PLAC1 antibody or anti-PLAC1-SN38 for 1 hr at 37°C, followed by washing and addition of goat anti-mouse IgG-HRP (1:2000; Bio-Rad, Hercules, CA, USA) at 37 °C for 1 hr. Signals were developed using Tetramethylbenzidine (TMB) and the optical density was measured at 450/620 nm by an ELISA reader.

Flow Cytometric Analysis of Anti-PLAC1-SN38 Reactivity

To assess whether SN-38 conjugation affected the reactivity of the anti-PLAC1 antibody, flow cytometric analysis was performed. MDA-MB-231 cells were detached at 70–80% confluence using saline sodium citrate buffer pH 8.0 (15 mM sodium citrate and 130 mM potassium chloride) at 37 ºC for 4 min. Detached cells were collected and washed three times with cold PBS containing 2% FBS. Cells were then incubated with either anti-PLAC1 antibody or anti-PLAC1-SN38 (5 µg/mL) for 45 minutes at 4 °C. Isotype controls, either unconjugated or conjugated with SN38, were used for gating. The "isotype control" employed in this study was a monoclonal anti-KLH antibody previously produced in our laboratory. KLH (Keyhole Limpet Hemocyanin) is a protein derived from the hemolymph of the keyhole limpet, a marine mollusk. Antibodies against KLH do not cross-react with human or mouse proteins, serving solely as a control in our experiments. Following three washes, cells were incubated with sheep anti-mouse IgG polyclonal antibody conjugated to FITC (1:100; Sina Biotech, Tehran, Iran) for 30 minutes in the dark. After an additional three washes, flow cytometric analysis was performed using a Partec flow cytometer (Munster, Germany). Data were analyzed using FlowJo software version 7.6.1.

Assessment of Anti-PLAC1-SN38 Cytotoxicity on MDA-MB-231 Cells

MDA-MB-231 cells were cultured as previously described. Twenty-four hours prior to the experiment, cells were detached and seeded into flat-bottom 96-well plates at a density of 5000 cells per well in a final volume of 100 µl culture medium, maintained in a humidified atmosphere (37 ºC, 5% CO2). Various concentrations of anti-PLAC1-SN38 (0.62-10 µg/ml), isotype antibody-SN38 (0.62-10 µg/ml) and equivalent concentrations of free SN38 (33-528 nM) were added to the wells. After 48 hours of incubation, cell viability was assessed using a WST-1-based colorimetric assay (Roche, Basel, Switzerland). Optical density was measured

at 450 nm against a background control (wells containing no cells) using a microplate ELISA reader. In our previous study, we demonstrated that the unconjugated anti-PLAC1 antibody alone had negligible effects on cell proliferation and cytotoxicity both *in vitro* and *in vivo* (14). Therefore, this control was omitted in the current experiments, and the isotype control antibody conjugated with SN38 was used instead.

Ex Ovo Chorioallantoic Membrane (CAM) Assay

Fertilized white Leghorn eggs were washed with warm water and disinfected by spraying with 75% ethanol. After drying, the eggs were incubated at 37°C with 70% humidity and automatically rotated using an Easy200 incubator (J.Hemel Brutgeräte). On embryonic day 3, the eggs were cracked and chick embryos were transferred into sterile weighing boats, covered with lids, and maintained at 37°C and 70% humidity. On embryonic day 10, 1×10^6 MDA-MB-231 cells, detached using citrate buffer and pre-incubated with either anti-PLAC1-ADC or isotype ADC for 1 hour at 37°C, were mixed with 5 µL of Matrigel® (BD Biosciences, #356237) and applied onto vascular branches of the CAM within sterile silicon rings (5 mm diameter). Tumors were allowed to develop for 3 days and subsequently excised along with the surrounding CAM tissue. Images were captured on day 0 and day 3 of tumor development using a Stereomicroscope SZX16 (Olympus).

Immunohistochemical Staining of Paraffin-embedded Tissues

CAM xenografts were excised and fixed in 4% paraformaldehyde overnight. Tissue dehydration was performed using a graded ethanol series followed by toluene treatment. Samples were then embedded in paraffin and sectioned. Hematoxylin and eosin (H&E) staining was conducted. Immunohistochemical (IHC) staining was performed as previously described (32). Briefly, after deparaffinization and rehydration, sections underwent antigen retrieval. primary antibodies used included anti-Ki67 (1:50; Agilent Technologies, Santa Clara, California, US) and polyclonal anti-rhPLAC1 antibody (100 ng/ml) (11). Detection was achieved using horseradish peroxidase (HRP) and diaminobenzidine (DAB) as the chromogen. Images of stained sections were captured using an Olympus BX53 microscope and analyzed with Olympus cell Sens Dimension 1.18 software (Olympus Corporation).

Pharmacokinetic Analysis of Anti-PLAC1-SN38

A pharmacokinetic study with anti-PLAC1-SN38 was conducted in four male NMRI mice obtained from the Avicenna Research Institute animal facility (Tehran, Iran). The anti-PLAC1-SN38 was sterilized using 0.2 µm membrane filters, and a single intravenous dose of 100 µg (dissolved in 166 µl PBS) was administered via the tail vein, corresponding to a dosage of 4 mg/kg. Blood samples were collected from the orbital venous sinus using sterile micro-hematocrit capillary tubes at multiple time points ranging from 15 min to 360 hours post-injection. To quantify serum concentrations of anti-PLAC1-SN38, an ELISA was developed A polystyrene 96-well plate was coated with 10µg/ml rhPLAC1 overnight at 4°C. Following standard washing and blocking procedures, wells were separately incubated either with two-fold serial dilutions of anti-PLAC1-SN38 (3.1-100 ng/ml) to generate a standard curve, or with serial dilutions of murine sera (1:100-1:800) for 1 hr at 37°C. After washing, signals development was performed as previously described. Serum concentrations of anti-PLAC1-SN38 were calculated based on the standard curve.

Assessment of in vivo Anti-tumor Effects of Anti-PLAC1-SN38 in MDA-MB-231 Xenografts

MDA-MB-231 cells were cultured as

previously described and harvested when they reached 70–80% confluence. Xenografts were established in nineteen female nude mice (8- to 10- week old) obtained from the Avicenna Research Institute animal facility. A total of 4×10^6 cells suspended in 100 µl of a 1:3 mixture of matrigel and cold serum-free DMEM were implanted subcutaneously into the mammary fat pad of each mouse. Treatment was initiated when the average tumor volume reached approximately 100 mm^3. Experimental and control groups (n=3 per group) received intraperitoneal injections of either anti-PLAC1-SN38 or isotype-SN38 at a dose of 4 mg/kg administered every 80 hours for a total of three doses. Tumor volumes were measured daily using a caliper. At the end of the study, mice were anesthetized with intraperitoneal injection of Thiopental sodium (40 mg/kg) to ensure deep unconsciousness. Euthanasia was subsequently performed using CO_2 gas. The absence of cardiac and respiratory activity was confirmed through direct observation to ensure the procedure was both effective and humane.

Angiogenesis Assessment in Chick Chorioallantoic Membrane Assay

Fertilized eggs were cracked and the chick embryos were cultivated as previously described. On day 10 of incubation, MDA-MB-231 cells were detached using citrate buffer and treated with either anti-PLAC1-SN38 (2 µg/onplant) or isotype-SN38 (2 µg/onplant) for 1 h. Treated cells were then suspended in MEM medium supplemented with 2 mg/ml collagen and 10 mM HEPES, kept on ice. Thirty microliters of the cell suspension were applied onto nylon grids and incubated for 30 min at 37 ºC. A total of 36 onplants (grid+ cell suspension) per group were carefully placed onto six eggs (six onplants per egg) and incubated for 3 days. Angiogenesis was evaluated by counting the number of blood vessels in the upper grid squares of six onplants per group under a microscope. The results were expressed as the average number of vessels per grid square.

Statistical Analysis

Statistical comparisons between groups were performed using the Mann-Whitney Rank Sum Test. Analyses were conducted using SigmaPlot software version 12.2.0.45. Statistical significance was considered to be present at $p<0.05$.

RESULTS

Assessment of the Binding Capacity of Anti-PLAC1-SN38 Using ELISA and Flow Cytometry

The anti-human PLAC1 monoclonal antibody (clone 2H12C12) was produced and characterized as previously described (14). Aminated SN38 (14) was conjugated to either anti-PLAC1 antibody or an isotype-matched irrelevant antibody. Conjugation was confirmed by ultraviolet–visible spectroscopy and HPLC (Fig. 1a and b). DAR was then calculated to be approximately 7.5. In HPLC analysis (Fig. 1b), the peak observed at 376.51 nm corresponded to the antibody-SN38 conjugate. Due to the small molecular size of SN-38, individual antibody molecules may incorporate variable numbers of drug moieties, resulting in a heterogeneous population of conjugates with a continuous distribution of DARs. Therefore, the reported DAR represents an estimated average number of SN-38 molecules conjugated per antibody.

Since conjugation of small molecules such as drugs to antibodies can potentially alter their binding activity through conformational changes (33), we assessed the binding capacity of anti-PLAC1-SN38 in comparison to the unconjugated antibody using ELISA and flow cytometry (Fig. 1). While ELISA revealed overlapping reactivity profiles between the naked and conjugated antibodies (Fig. 1c), flow cytometry analysis showed a modest but statistically significant enhancement in the binding of the conjugated antibody to the native antigen ($p<0.05$; Fig. 1d).

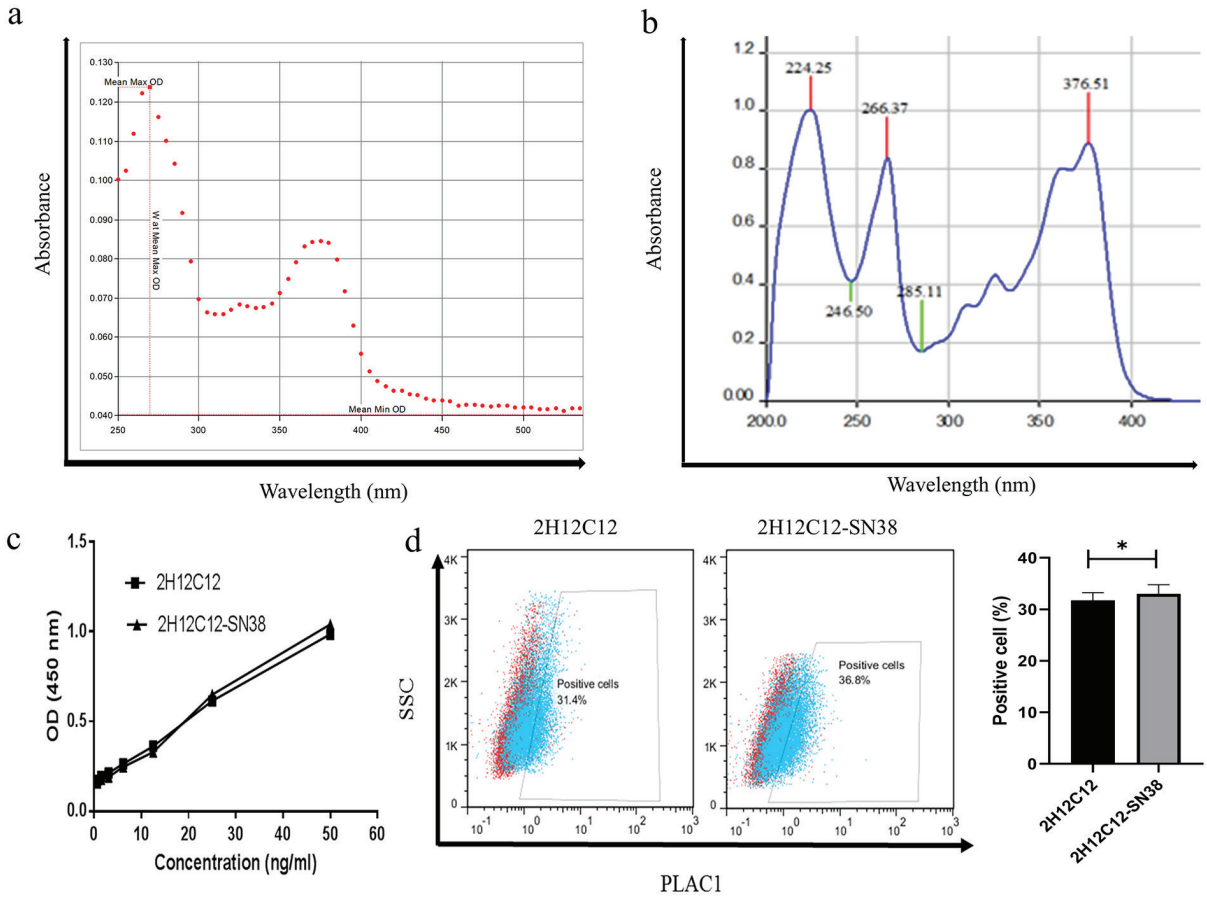

Fig. 1. Conjugation of SN38 to anti-PLAC1 antibody and assessment of anti-PLAC1-SN38 reactivity by ELISA and flow cytometry: (a) Conjugation of SN38 to anti-PLAC1 was confirmed using ultraviolet–visible spectroscopy and (b) high-performance liquid chromatography (HPLC) (c) The reactivity of anti-PLAC1-SN38 conjugate against rhPLAC1 was compared to that of the unconjugated antibody using ELISA. (d) Flow cytometric analysis was performed on MDA-MB-231 cells to assess the binding of SN38-conjugated anti-PLAC1 antibody relative to the intact antibody. Although ELISA revealed overlapping reactivity profiles between the naked and conjugated antibodies, flow cytometry demonstrated a modest but statistically significant increase in binding of the conjugated antibody to the native antigen (*p<0.05).

In vitro Cytotoxicity Profiling of Anti-PLAC1-SN38 on MDA-MB-231 Cells

The cytotoxic potential of anti-PLAC1-SN38 was evaluated in MDA-MB-231 cells using WST-1 cell proliferation assay. Cells were treated with free SN38, SN38-labeled isotype control, or anti-PLAC1-SN38. The concentration of free SN38 was matched the amount of SN38 conjugated to the ADCs. Free SN38 exhibited minimal cytotoxicity up to 264 nM, with only a slight reduction in cell viability. In contrast, anti-PLAC1-SN38 induced substantial cytotoxic effects across all tested concentrations in a dose-dependent manner. PBS and wells without cells served as negative and positive controls, respectively (Fig. 2a).

Pharmacokinetics of Anti-PLAC1-SN38 in Mouse Serum

Pharmacokinetic characterization of ADCs reflects the dynamic interplay between the biological system and the therapeutic agent. ADCs often exhibit accelerated clearance and reduced half-lives compared to their unconjugated counterparts (34-36), likely due to conformational changes affecting their tertiary structure. To determine the half-life of anti-PLAC1-SN38 in mouse serum, the conjugate was administered intravenously at a dose of 4 mg/kg per mouse, and its concentration was quantified in serum samples collected at multiple time points using ELISA. The detection range for this assay was 10-100 ng/ml (Fig. 2b).

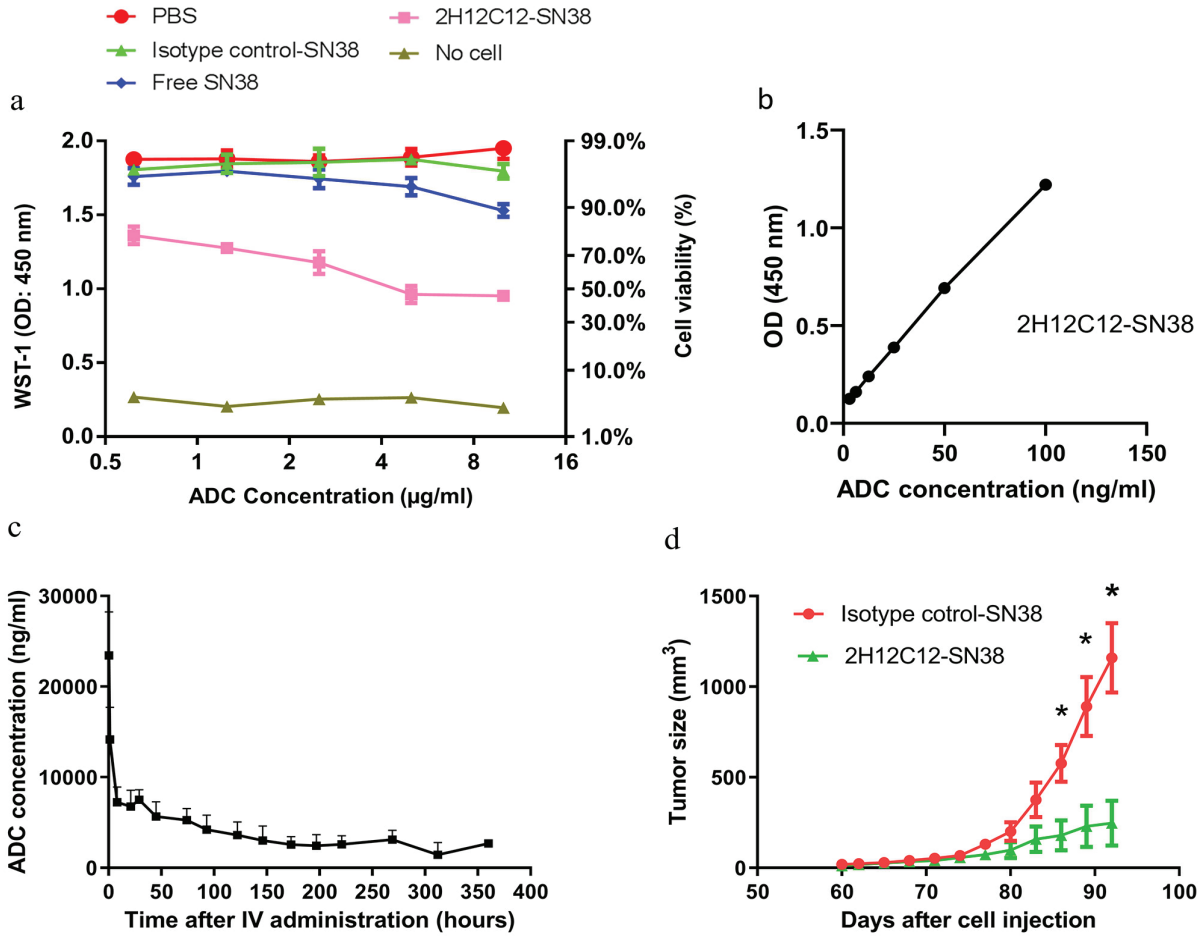

Fig. 2. *In vitro* cytotoxicity, pharmacokinetic profile, and *in vivo* anti-tumor efficacy of anti-PLAC1-SN38. (a) MDA-MB-231 cells were treated with equivalent concentrations of free SN38, SN38-labeled isotype control, or anti-PLAC1-SN38, and cytotoxicity was assessed using the WST-1 cell proliferation assay. Free SN38 showed negligible cytotoxicity up to 264 nM, whereas anti-PLAC1-SN38 exhibited significant dose-dependent cytotoxicity. PBS (negative control) and wells without cells (background control) were included for normalization. (b) Standard curve was plotted for quantification of anti-PLAC1-SN38 in mouse serum (detection range: 10–100 ng/mL) by ELISA. In this assay, wells were coated with rhPLAC1 and reactivity of anti-PLAC1-SN38 with coating layer was then assessed. (c)Pharmacokinetic analysis of serum concentrations of anti-PLAC1-SN38 following intravenous administration (4 mg/kg) was performed at multiple time points using the standard curve. The ADC exhibited an estimated half-life of approximately 80 hours, consistent with reported pharmacokinetic profiles of antibody-drug conjugates. (d) Mice bearing MDA-MB-231 tumors were treated intraperitoneally three times with either isotype-SN38 or anti-PLAC1-SN38 at a dose of 4 mg/kg every 80 hours. Tumor volumes were measured daily using caliper. Treatment with anti-PLAC1-SN38 significantly suppressed tumor growth compared to the control (*p<0.05 on days 86, 89, and 92)

The temporal profile of anti-PLAC1-ADC serum concentrations is depicted in Fig. 2c. Our data demonstrated a sharp decline in ADC levels within 15 minutes post-administration, followed by a more gradual decrease over the duration of the study. Based on these results, the average half-life (t1/2) of anti-PLAC1-SN38 in mouse circulation was approximately 80 hours. This value aligns with the reported half-life range of ADCs, which typically spans from 2 to 10 days (37, 38).

Inhibitory Effect of Anti-PLAC1-ADC on Tumor Growth in vivo

MDA-MB-231 xenografts were established in female nude mice, with a tumor incidence of 31% and a latency period of 60 days. Treatment was initiated when the average tumor volume reached approximately 100 mm^3.

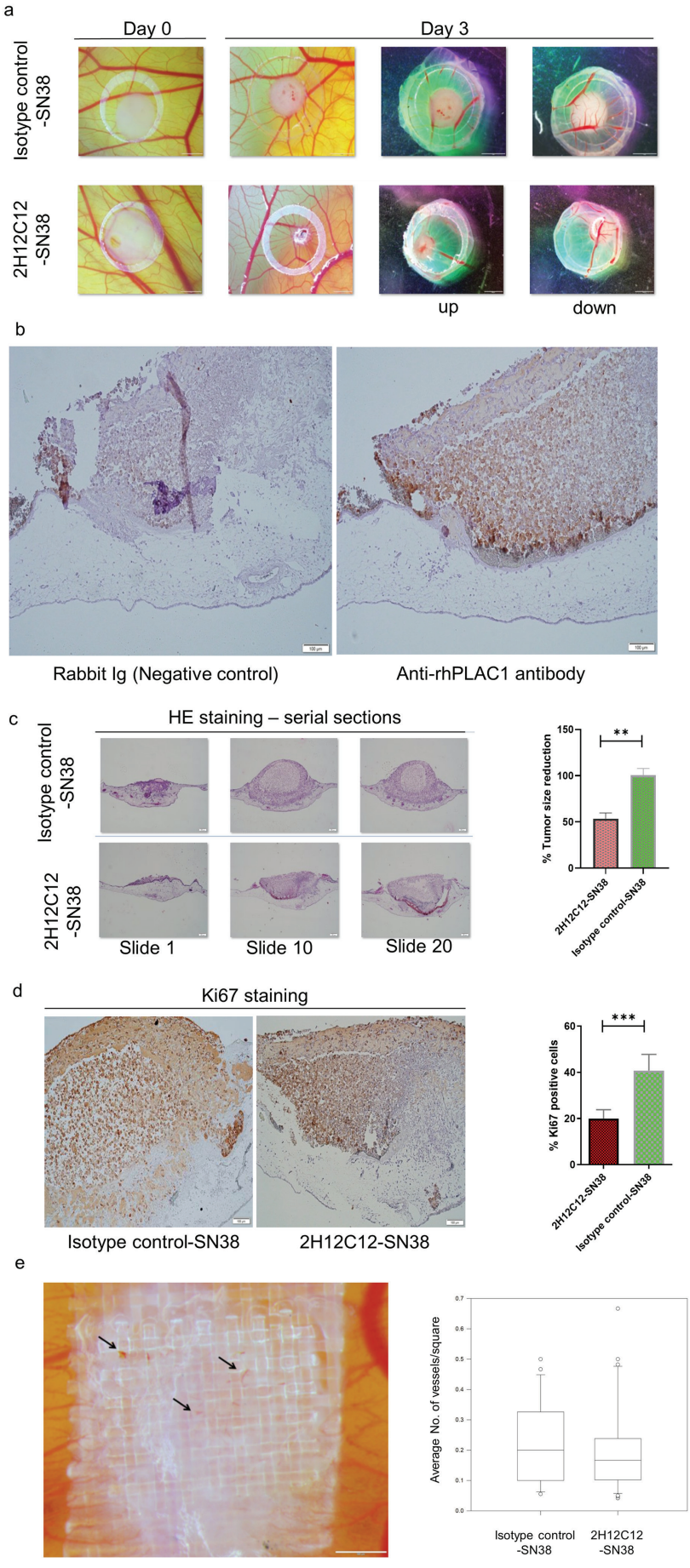

Fig. 3. Assessment of the tumor inhibitory effect of anti-PLAC1-SN38 in the chorioallantoic membrane (CAM) assay. (a) MDA-MB-231 cells, treated with either anti-PLAC1-SN38 or isotype control-SN38, were grafted on CAM, and xenografts were photographed on days 0 and 3. Additionally, tumors were excised and imaged from both top and bottom views on the specified days. (b) PLAC1 protein expression on CAM-derived tumors was evaluated using immunohistochemistry. (c) Hematoxylin and Eosin staining was performed on every 10th section of the CAM tumor samples. Treatment with the anti-PLAC1-SN38 conjugate resulted in significantly smaller tumors compared to those treated with the non-specific isotype control-SN38 (**p<0.01). (d) Tumor cell proliferation was assessed by Ki67 immunostaining. MDA-BB-231 cells treated with anti-PLAC1-SN38 exhibited a significantly lower proliferation index than the control group (***p<0.001). (e) Angiogenesis was evaluated by counting tumor-associated vessels, indicated by arrows, within the upper grid of each onplants. Statistical analysis using the Mann–Whitney Rank Sum test showed no significant difference in angiogenesis between tumors treated with anti-PLAC1-SN38 and those treated with isotype control-SN38 (p=0.382) (e).

Both anti-PLAC1-SN38 and isotype control-SN38 were administered intraperitoneally 80-hours intervals. Mice treated with anti-PLAC1-SN38 exhibited a statistically significant reduction in tumor volume compared to those receiving isotype control-SN38 on days 86 (p=0.0385), 89 (p=0.0290), and 92 (p=0.0163) (Fig. 2d). In a comprehensive safety assessment conducted by this research group (14), the in vivo toxicity profile of anti-PLAC1-ADC was evaluated through histopathological examination of the lung, liver, kidney, spleen, stomach, and brain in treated animals. The findings indicated no detectable microscopic abnormalities in any of the examined tissues, supporting the safety of this ADC formulation.

Inhibitory Effect of Anti-PLAC1-SN38 on MDA-MB-231 Tumor Growth in CAM Assay

To evaluate the inhibitory effect of anti-PLAC1-SN38, MDA-MB-231 cells pre-treated with either anti-PLAC1-SN38 or isotype control-SN38 were grafted onto the CAM. Xenograft images were captured on days 0 and 3 (Fig. 3a). Immunohistochemical analysis of the resulting tumors confirmed sustained PLAC1 expression in the CAM model (Fig. 3b). Tumors exposed to anti-PLAC1-SN38 were significantly smaller than those treated with isotype-SN38 (p=0.008, Fig. 3c). Additionally, cellular proliferation was assessed via immunohistochemistry using an anti-Ki67 antibody. Five representative regions per tumor section were analyzed, and Ki-67-positive cells were quantified. The results demonstrated significantly reduced proliferation in anti-PLAC1-SN38-treated tumors compared to the isotype control-SN38 group (p=0.0003, Fig. 3d).

Assessment of the Effect of Anti-PLAC1-SN38 on Tumor Angiogenesis in the CAM Assay

The impact of anti-PLAC1-SN38 on angiogenesis in MDA-MB-231 tumors was investigated by CAM assay (39). Tumor-associated vasculature quantified by counting vessels within the upper grid of each onplants across treatment groups. Statistical analysis using Mann-Whitney Rank Sum test revealed no significant difference in angiogenesis between tumors treated with anti-PLAC1-SN38 and those receiving isotype control-SN38 (p=0.382, Fig. 3e). The lack of anti-angiogenic activity, despite a reduction in tumor size suggests that anti-PLAC1-SN38 exerts its therapeutic effect primarily through direct cytotoxicity against tumor cells rather than through inhibition of neovascularization.

DISCUSSION

Conventional cancer therapies are often associated with off-target cytotoxicity affecting normal cells, whereas immunotherapeutic approaches generally exhibit reduced collateral damage. Although certain monoclonal antibodies- such as anti-HER2- or anti-CD20-have demonstrated substantial anti-cancer efficacy, recent investigations indicate that most monoclonal antibodies with specific antigen-specific binding capacity possess limited anti-tumor activity (15, 40, 41). Despite the limited standalone activity of monoclonal antibodies, their high specificity for cancer- associated antigens makes them valuable anti-tumor agents. The specificity can be harnessed to effectively target cancer cells, particularly when antibodies are conjugated with cytotoxic agents (42). At present, more than 550 active clinical trials investigating ADCs are ongoing worldwide (43). The primary concern in employing antibodies for cancer therapy is the specificity of the targeted antigens to cancer cells. PLAC1 belongs to the cancer-testis-placental antigen family and exhibits distinct characteristics that make it a promising candidate for cancer immunotherapy. Its limited expression in normal tissues, broad expression across multiple cancer types of diverse histological origins, surface localization, and functional role in tumor development and progression represent key features of an ideal therapeutic

target (16-20). We previously demonstrated that anti-PLAC1 antibodies specifically bind to their cognate antigen but do not induce cytotoxicity in cancer cells (14, 44). In contrast, SN38- conjugated anti-PLAC1 antibody showed substantial cytotoxic activity against prostate and melanoma cancer cell lines.

SN-38, the active metabolite of irinotecan, is a topoisomerase I inhibitor that has become a highly effective payload for ADCs due to its exceptional potency, well-defined mechanism of action, and favorable physicochemical properties. Compared with conventional chemotherapeutic agents such as irinotecan, SN38 is 100 to 1,000 times more potent (45). Its half-maximal inhibitory concentration (IC_{50}) lies in the sub-nanomolar range, enabling highly cytotoxic activity at very low intracellular concentrations (46). This property is particularly advantageous for ADCs, as only a small fraction of the administered dose reaches the tumor site. Additional benefits of SN-38 include broad-spectrum antitumor activity and the ability to overcome drug resistance mechanisms (47). SN38 has been extensively utilized as a payload in ADCs (3). Antibodies conjugated with SN38 have been developed to target diverse tumor types (12) by inhibiting topoisomerase I activity (2).

In the present study, we investigated the anti-cancer effects of an anti-PLAC1 antibody and its SN38-based ADC in breast cancer using *in vitro*, *ex vivo*, and *in vivo* models. We demonstrated that conjugation of anti-PLAC1 antibody with SN38 did not compromise its antigen-binding activity, which is a crucial prerequisite for effective ADC design. Our previous work showed that SN38-labeled anti-PLAC1 antibodies were rapidly internalized into prostate cancer cells upon binding to PLAC1, leading to selective cytotoxicity in PLAC1-expressing cells without off-target effects (14). Consistent with these findings in prostate and melanoma cancers (14, 44), we now show that the anti-PLAC1-SN38 conjugate exerted a specific cytotoxic effect on MDA-MB-231 breast cancer cells *in vitro*, with the magnitude of cell killing corresponding to the proportion of PLAC1-expressing cells.

In vivo, the therapeutic efficacy of the anti-PLAC1-SN38 ADC was evaluated in MDA-MB-231 tumors using both CAM assays and nude mouse models. In these experiments, the anti-PLAC1 ADC significantly inhibited tumor growth. However, it did not affect angiogenesis within MDA-MB-231 tumors, indicating that its anti-tumor activity is not mediated through anti-angiogenic mechanisms.

Although our study demonstrated approximately 30% surface expression of PLAC1 on MDA-MB-231 cells, multiple reports have confirmed PLAC1 is localized both intracellularly and on the cell surface (10, 44, 48-51). This dual localization is highly relevant for antibody-based cancer immunotherapy. Apoptotic bodies generated following ADC-induced cell death can be taken up by antigen-presenting cells, potentially eliciting anti-PLAC1 cytotoxic T lymphocyte (CTL) responses that amplify the therapeutic efficacy of the ADC. (44). This mechanism may account for the greater-than-expected therapeutic effect of the anti-PLAC1 ADC observed in our study. Consistent with this, we have recently demonstrated the immunoenhancing role of intracellular plac1 in a mouse model of melanoma (27). Similarly, despite PLAC1 expression being detected in only ~30% of MDA-MB-231 cells, we observed that tumors in anti-PLAC1 ADC-treated mice were at least fivefold smaller than those in the control group. However, because nude mice lack functional T cell–mediated immunity, the immunoenhancement potential of intracellular PLAC1 is unlikely to account for this effect in this model. This finding suggest that PLAC1 expression may be regulated by the tumor microenvironment, where signals from tumor-associated cells and the extracellular matrix could promote upregulation of PLAC1. Supporting this notion, we recently demonstrated that PC3

prostate cancer cells grown as spheroids exhibited considerably increased surface PLAC1 expression (52). Taken together, although isolated cancer cells display relatively low surface PLAC1 expression, our results indicate that anti-PLAC1 ADC can nonetheless exert potent therapeutic effects. This effect could be attributed to two factors: (1) tumor microenvironment-mediated upregulation of PLAC1 and (2) the immunostimulatory potential of intracellular PLAC1—although the latter would only be relevant in immunocompetent models. Given the established success of immune checkpoint inhibitors in several malignancies, their combination with anti-PLAC1-ADC therapy may further enhance therapeutic efficacy in cancer immunotherapy.

The limitations of this study include the lack of detailed mechanistic insights into ADC function both *in vitro* and *in vivo* settings. Additionally, the relatively small number of nude mice used in the *in vivo* experiments may restrict the generalizability of the findings. Future studies with larger cohorts and more comprehensive mechanistic analyses will be necessary to validate and extend these results. Although parallel experiments comparing murine and human cell lines in immunocompetent versus immunocompromised models could theoretically enhance translational relevance, the inherent differences in PLAC1 biology between species and methodological constraints limit the feasibility of such approaches.Accordingly, we focused on evaluating the anti-human PLAC1 ADC in nude mice bearing MDA-MB-231 xenografts, while recognizing the limitations of this model, including tumor-TME incompatibility, absence of immune components, and low implantation rates.

CONCLUSION

Our study provides the first evidence of the therapeutic efficacy of a PLAC1-ADC in a breast cancer mouse model, underscoring the potential of PLAC1-targeted immunotherapy as a promising strategy for human breast cancer.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All experimental procedures were approved by the Ethics Committees of the Avicenna Research Institute (Approval No. 960511-036) and Tehran University of Medical Sciences (Approval No. 27939-57-01-94). Ethics approval and consent to participate

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AUTHORS' CONTRIBUTION

J.M. contributed to performing experiments, data analysis, and writing the article. R.GZ. and M.R.N. performed experiments. R.G, M.J.T., N.G., and S.N.O. contributed to data interpretation. A.H.Z. contributed to the study design, data interpretation, and writing the article.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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