Preclinical studies of a Pro-antibody-drug conjugate designed to selectively target EGFR-overexpressing tumors with improved therapeutic efficacy

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\textbf{ABSTRACT}

Antibody-drug conjugates (ADCs) have exhibited potent clinical benefits in cancer therapy. However, development of ADCs against epidermal growth factor receptor (EGFR) has limitations because of wide expression of EGFR in both normal and tumor tissues. Previously, we developed an anti-EGFR protease-activated antibody (pro-antibody), termed as PanP, which remains inert against EGFR until activated by tumor-specific protease. Herein, we for the first time report a new class of pro-antibody-drug conjugate (PDC) against EGFR, denoted as PanP-DM1. It has been designed to selectively target the EGFR-overexpressing tumor cells and exert greater anti-tumor activity compared with PanP. Our data showed that PanP-DM1 also could be selectively activated by tumor-specific protease ‘uPA’. Furthermore, activated PanP-DM1 was potently cytotoxic against EGFR-overexpressing tumor cell lines in vitro. Crucially, our data indicated that PanP-DM1 was significantly more effective in eradicating EGFR-overexpressing tumors in vivo. Additionally, toxicity was preliminarily evaluated in mice as measured by body weight loss. In summary, our study suggests that PanP-DM1, a novel pro-antibody-drug conjugate, has cancer-selectivity, efficacy and safety profile that supports its potential use for EGFR-overexpressing tumors.

\textbf{Abbreviations:} ADC, antibody–drug conjugate; CRC, colorectal cancer; CCK-8, Cell Counting Kit 8; DAR, drug to antibody ratio; EGFR, epidermal growth factor receptor; Endo F2, Endo-β- N-acetylgalcosaminidase F2; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; mAb, monoclonal antibody; Pro-antibody, protease-activated antibody; PDC, Pro-antibody-drug conjugate; RP-UPLC, Reverse-phase Ultra Performance Liquid Chromatography; SMCC, N-succinimidyl-4-[maleimidomethyl]-cycllohexane carboxylate; SEC, size-exclusion chromatography.

\textbf{Introduction}

Epidermal growth factor receptor (EGFR), a transmembrane receptor kinase, plays a pivotal role in tumor progression. Aberrant EGFR activation is associated with tumorigenesis and metastasis.\textsuperscript{1} Porebska et al revealed its increased expression in 60–80% of colorectal cancer (CRC) cases.\textsuperscript{2} Furthermore, EGFR expression could be a prognostic marker in many cancers like CRC and breast cancer.\textsuperscript{3} More importantly, its localization on the cancer cell surface makes it an ideal molecular target for developing EGFR-directed antibodies.\textsuperscript{4} They specially bind to the extracellular domain III of EGFR, thereby blocking the ligand-binding domain and hindering the extended conformation of the dimerization arm on domain II.\textsuperscript{5,6} The US Food and Drug Administration (FDA)-approved antibodies against EGFR, cetuximab (Erbitux\textsuperscript{®}) and panitumumab (Vectibix\textsuperscript{®}), are routinely used and produce substantial therapeutic benefits in the treatment of KRAS wild-type advanced CRC.

Although EGFR-blocking antibodies have shown potent clinical efficacy, on-target skin toxicities associated with EGFR inhibition lead to interruption or dose modification, and affect patients’ quality of life.\textsuperscript{7,8} As reviewed by relevant studies, EGFR inhibitors were thought to affect keratinocytes by inducing skin inflammation and innate host defense.\textsuperscript{9,10} A protease-activated antibody (Pro-antibody) that is inactive in normal tissues and selectively activated by the proteases upregulated in tumor tissues is an attractive approach to reduce side effects caused by target binding in healthy tissues.\textsuperscript{11,12} Recently, Desnoyers et al designed a pro-antibody based on cetuximab that improved the safety profile without compromising the pre-clinical efficacy.\textsuperscript{13}
Antibody–drug conjugates (ADCs) are emerging as powerful anti-tumor therapeutics that combine tumor-targeted antibodies with active cytotoxic agents. Generally speaking, the ADC components consist of an antibody that targets internalized cell surface molecule and a highly cytotoxic compound. Numerous studies indicated maytansinoid DM1 (derivative of maytansine), a highly potent microtubule polymerization inhibitor, was an ideal payload for developing ADC. Furthermore, antibody-DM1 conjugates have shown promising results in preclinical and clinical evaluations. As a member of the EGFR family, HER2 is a clinically validated ADC target. The FDA-approved HER2-directed ADC, ado-trastuzumab emtansine (T-DM1), is composed of trastuzumab and DM1 for treating patients with HER2-positive breast cancer. Previous studies demonstrated that EGFR appears to be rapidly internalized when incubated with anti-EGFR antibodies such as panitumumab. An EGFR-targeted ADC, IMGN289 is currently being evaluated in a Phase 1 clinical trial. Therefore, an EGFR-targeted ADC may be a promising therapeutic, although it potentially increases the severity of the side effects that will be systematically evaluated in clinical trials. Notably, pro-antibody-derived drug conjugates against EGFR that combine the advantage of the pro-antibody’s target specificity with a drug’s cytotoxicity have not been reported yet. The properties of pro-antibody-drug conjugates (PDCs) should limit the toxicity on normal tissues.

Here, we developed a novel PDC against EGFR, designated PanP-DM1. Previously, we constructed and characterized the cancer-selective pro-antibody termed as PanP. It was engineered by fusing the peptide comprising uPA substrate sequence, blocking peptide and Gly-Ser–rich peptide linkers to the light chain N terminus of Pan that derived from panitumumab. In the present study, the maytansine (DM1) was conjugated to PanP through the stable non-reducible thioether linkage. The enhanced anti-tumor effects were assessed using in vitro and in vivo models. Further, we confirmed that PanP-DM1 could be internalized and induce cell cycle arrest. In addition, a preliminary toxicity study was performed in BALB/c mice and tumor-bearing nude mice by comparing the changes on body weight with injection of PanP-DM1. To conclude, these data suggest that PanP-DM1, the first cancer-selective PDC for EGFR-targeted therapy, holds promise for clinical development because of its high potency and improved cancer selectivity.

Results

Characterization of PanP-DM1

PanP-DM1, a conjugate where lysine residues were modified with DM1 via a non-reducible thioether linker, succinimidyl-4-[maleimidomethyl]-cyclohexane carboxylate (SMCC), was prepared as described in Materials and Methods. Schematic representation of PDC was shown in Fig. 1A. The resulting PanP-DM1 was firstly characterized by SDS-PAGE (Fig 1B). Under reducing conditions, the heavy chain of PanP-DM1 had a slightly higher molecular weight than that of PanP, suggesting that the linker drug preferentially attaches to lysine residues in the heavy chain as previously reported. In addition, only little aggregation was observed at non-reducing conditions; the content was then determined to be below 3% using size-exclusion chromatography (Fig S1).

Moreover, drug-to-antibody ratio (DAR) of PanP-DM1 was determined to be 4.2 using UV/VIS spectroscopic analysis, which is similar to that of commercial T-DM1. To further evaluate the drug distribution, deglycosylated PanP-DM1 was analyzed by ESI-T0F-MS. As illustrated in Fig. 1C, the spectrum shows 8 prominent peaks, which are equally spaced and correspond to antibody linked to zero to 7 DM1 molecules. Taken together, these data showed that PanP-DM1 has been successfully constructed in structure.

PanP-DM1 displays comparable property to PanP

An enzyme-linked immunosorbent assay (ELISA) was performed to investigate whether PanP-DM1 still maintains the binding properties of PanP. PanP-DM1 showed slightly reduced EGFR binding compared to PanP, suggesting that conjugation apparently did not affect the antigen binding (Fig 2A). It is important to note that PanP-DM1 displayed 12-fold weaker binding to immobilized EGFR than Pan (parental antibody of PanP) DM1 conjugate, indicating that PanP-DM1 retained masked binding of PanP.

To further evaluate the binding of uPA-activated PanP-DM1 on cell-surface EGFR, we used a fluorescence-activated cell sorting (FACS) assay. Activated PanP-DM1 was obtained by incubating PanP-DM1 with recombinant uPA in vitro as described previously and characterized using SDS-PAGE (Fig S2). Activated PanP-DM1 and activated PanP exhibited mostly equivalent ability to bind to EGFR-overexpressing A431 cells at saturating (10 μg/ml) and subsaturating (1 μg/ml) concentrations, suggesting that attachment of DM1 did not affect the uPA-mediated activation (Fig 2B). Moreover, PanP-DM1 showed about an order of magnitude increase in binding activity after exposure to uPA as determined by ELISA and FACS, which is comparable to Pan-DM1 (Fig 2C; Fig S3; Table S1). Thus, these results revealed that PanP-DM1 remains masked against EGFR binding until activated by uPA, which is similar to PanP.

PanP-DM1 has superior in vitro anti-tumor activity compared with PanP

Next, we compared the inhibitory effects of PanP-DM1, activated PanP-DM1 and activated PanP on 3 EGFR-overexpressing cancer cell lines (A431, H292 and DiFi cells). After 48 or 72h incubation, activated PanP-DM1 inhibited the growth of DiFi, H292 and A431 cancer cells in a dose-dependent manner (Fig 3A). Importantly, the growth inhibition was significantly stronger compared with activated PanP at the concentration of 1 μg/ml (P<0.001). Furthermore, as expected, activated PanP-DM1 exhibited good inhibitory activity compared to non-activated PanP-DM1 against these cells at a low concentration (0.1 μg/mL), indicating the functionally masked effect in the intact PDC (Fig 3B). These results demonstrated that PanP-DM1 had superior in vitro anti-tumor activity compared with PanP.
In addition, non-targeted inhibitory effects of PanP-DM1 conjugate were also evaluated on breast cancer cell lines expressing low levels of EGFR (BT-474 and MCF7). As a result, activated PanP-DM1 only showed minimal dose-dependent inhibitory activity in these cell lines (Fig 3C), which is equal to that of a control ADC, anti-tumor necrosis factor (TNF) mAb-DM1. Only activated PanP-DM1 at a high concentration (1 μg/ml) inhibited the growth of BT-474 or MCF-7 cells, indicating a non-specifically inhibitory effect.

Effect of PanP-DM1 on A431 cell cycle arrest

To further evaluate PanP-DM1s mechanism of action, we analyzed the conjugate-induced cell cycle arrest on A431 cells. Internalization of activated PanP-DM1, which is a necessary process for delivering DM1 into cells and triggering cell cycle arrest, was first evaluated in A431 cells. The surface level of activated PanP-DM1 markedly decreased on cells when shifted to 37°C over the course of 90 minutes study (Fig 4A), suggesting rapid internalization of the conjugate into A431 cells.

The effect of activated PanP-DM1 on cell cycle was subsequently determined by flow cytometry. Treatment with activated PanP-DM1 resulted in the remarkable G2/M phase arrest, which may directly lead to cell apoptosis (Fig 4B and C). The percentage of cells in the G2/M phase increased from 19.69 ± 1.809% to 30.92 ± 1.097% in activated PanP-DM1-treated culture and from 19.69 ± 1.809% to 25.92 ± 1.092% in PanP-DM1-treated culture (Fig 4C). These results revealed that non-activated PanP-DM1 was less effective than activated PanP-DM1 in inducing G2/M arrest on A431 cells (P<0.05), which is likely attributable to reduced antigen-binding ability. In addition, in line with previous studies, activated PanP induced G0/G1 arrest, which is a known mechanism of action of panitumumab and cetuximab (Fig 4B). Together, these data implied that PanP-DM1 exerts enhanced anti-tumor effect through targeted delivery of DM1 and inducing G2/M cell cycle arrest.
Therapeutic effects of PanP-DM1 in A431 and H292 xenograft tumor models

Next, the therapeutic effects of PanP-DM1 and PanP were examined in nude mice bearing established A431 and H292 xenograft tumors. The two cell lines can express and secrete uPA as previously described.38,39 As shown in Fig. 5A, both PanP and PanP-DM1 were effective in delaying the A431 xenograft tumor progression. It is particularly noteworthy that treatment with PanP-DM1 resulted in rapid and nearly complete tumor regression from day 7 post injection, whereas only partial tumor growth inhibition was observed in tumor-bearing mice treated with PanP. At the end of observation period (day 21), tumors were absent in all mice (n = 10) treated with PanP-DM1, whereas mice treated with PanP already reached tumor volume of 555.8 ± 70.58 mm³. Evidently, PanP-DM1 inhibited xenograft tumor progression. 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tumor growth much more effectively than PanP in A431 tumor models.

We then confirmed the anti-tumor effect of PanP-DM1 in the H292 xenograft tumor model (Fig 5B). A single intraperitoneal (i.p.) injection of PanP-DM1 at 20 mg/kg achieved complete tumor regression in all mice (n = 10). Notably, PanP-DM1 inhibited tumor development more effectively than PanP, although the difference was less pronounced than on faster growing A431 tumors. In conclusion, consistent with in vitro results, PanP-DM1 also exhibited significant benefit over PanP in the A431 and H292 xenograft tumor models.

**Evaluation of PanP-DM1’ non-specific toxicity in mice**

To assess the therapy-related unspecific toxicity on PanP-DM1 treatment, body weight was monitored in nude mice bearing established subcutaneous A431 and H292 tumor xenografts. For A431 tumor-bearing mice, treatment with PanP-DM1 was well tolerated and the average body weight markedly recovered after marginal weight loss post the second injection (Fig 6A). Similar results were also observed on H292 tumor-bearing mice treated with PanP-DM1. Over the study period (about 18 days), these mice did not significantly lose body weight and behaved normally.
Figure 6. Toxicity and tolerability evaluation of PanP-DM1 in mice. (A) Effect of PanP-DM1 on nude mice body weight was determined using A431 or H292 tumor-bearing nude mice. Mice were weighed at regular intervals during the whole period to monitor therapy-related toxicity. (B) Body weight change of BALB/c mice on treatment with different dose schedules of PanP-DM1. Mice were randomly divided into groups of 5 mice each and treated on day 0 with a single i.p. dose of PanP-DM1 (0, 25, 50, 75 or 100 mg/kg). Changes in body weight over time relative to day 0 were plotted. Arrows indicate the time point of injection. Data are shown as means ± SEM. **P < 0.01, ***P < 0.001.

As PanP does not recognize mouse EGFR, the antigen-independent effect of PanP-DM1 was measured on BALB/c mice by body weight loss at multiple doses (Fig 6B). Of the tested dose, PanP-DM1 treatment at 0, 25 or 50 mg/kg did not exhibit body weight loss. However, administration of PanP-DM1 at a dose of 75 or 100 mg/kg resulted in considerable transient body weight loss (-8% or -12%) in the first 3 days, indicating that a single-dose of PanP-DM1 above 75 mg/kg exerted a toxic effect on BALB/c mice. Overall, PanP-DM1 at multiple doses appears to be well tolerated.

Discussion

To our knowledge, this is the first study to describe a PDC against EGFR. Our previous study revealed that PanP has enhanced anti-tumor potency and target selectivity. In the present study, our data showed that PanP-DM1 retained the properties of PanP and was much more active in eradicating established tumors. As noted in our previous study, 50 mg/kg dose of PanP (2 times per week) for 3 weeks ultimately eradicated established A431 tumors in tumor-bearing mice. Strikingly, 2 doses of 35 mg/kg PanP-DM1 conjugate in one week resulted in complete regressions in A431 tumor-bearing mice, suggesting its potential advantage for clinical therapy.

Generally speaking, conjugates with lesser or higher drug load are likely to result in compromised anti-tumor effects or increased systemic toxicities. PanP-DM1, with a DAR of 4.2:1, has exhibited improved therapeutic effect and was well tolerated in mice, demonstrating the ratio seemed to be favorable for developing PDC against EGFR. A previous study reported that the paclitaxel-cetuximab conjugate has enhanced cytotoxicity compared to intact antibody in vitro. However, the lack of a significant difference between cetuximab and the conjugate was found in the in vivo model, which may be explained by insufficient dose of mAb-delivered paclitaxel or untimely release of it. Therefore, we chose the stable thioether linker SMCC and highly potent agent DM1 to construct PanP-DM1. As a result, it has shown strong anti-tumor potency in vitro and in vivo, indicating that the linker and cytotoxic drug play important roles in governing the efficacy and viability of conjugates.

Skin toxicities were observed in patients after treatment with anti-EGFR antibodies. ADCs targeting EGFR could potentially increase the severity of the side effects. AMG 595, an ADC directed to the tumor-specific antigen EGFRvIII was designed to minimize on-target skin toxicities that might be observed in ADCs against wild-type EGFR. In addition, PDCs that could be selectively activated in tumors have the potential to limit on-target skin toxicities without compromising the therapeutic effect. Data shown here revealed that PanP-DM1 exhibited attenuated functional activities on antigen binding, cell growth inhibition and cell cycle arrest, but, when activated by uPA, it showed significantly enhanced activities in these assays. A previous study concluded that stronger binding clearly leads to higher toxicities among EGFR-targeted antibodies. Thus, PanP-DM1 has the potential to achieve desired tolerability profile with less binding on normal cells than other conventional anti-EGFR ADCs.

Furthermore, the toxicity evaluation provides a valuable reference for further safety assessment of PDC in cynomolgus monkeys. Notably, PanP-DM1 eradicated A431 and H292 xenografts in mice with no obvious side effects at the effective doses. Since PanP-DM1 has no cross-reactivity with mouse tissues, the potential safety benefits conferred by cancer-selectivity of PanP-DM1 could not be sufficiently evaluated in our present study. And further studies will be focused on evaluating the on-target cutaneous toxicity in nonhuman primates.

Drug resistance with the current established anti-EGFR antibodies such as cetuximab creates the need for better therapeutic avenue. ADC has become a powerful treatment strategy to overcome drug resistance like trastuzumab resistance. The efficacy of PanP-DM1 on the cancer cells that are refractory to EGFR-directed antibodies will be explored in a future research publication.

Collectively, the data presented here suggest that PanP-DM1 has the potential not only to dramatically improve the therapeutic effect of PanP, but also to exhibit cancer-selective activity. Therefore, development of PDC provides a new strategy for targeting EGFR in a cancer-specific manner, which contributes to enhancing therapeutic effect and improving the safety index over conventional ADC.

Materials and methods

Cell lines, animals and other reagents

The human epithelial carcinoma cell line A431, human breast cancer cell lines MCF-7 and BT-474 and human lung cancer cell line NCI-H292 were obtained from the American Type Culture Collection (ATCC). The human colorectal cancer cell
The assay was performed as described previously. Brie-egg ELISA and Flow Cytometry was used to analyze the obtained mass spectrum.

Resolved on 10% SDS PAGE under reducing conditions and nonreducing conditions, followed by Coomassie brilliant blue staining.

**Conjugation and characterization of pro-antibody-drug conjugate (PDC)**

DM1 conjugated PanP, termed PanP-DM1, was generated by conjugation of DM1-SMCC on antibody lysine groups as described in US 2011/003969 A1. In brief, antibody at 5 mg/ml was mixed with ~6.4 mg/ml DM1-SMCC in a phosphate buffer (50mM, pH = 7.5) containing 2mM EDTA and 50 mM NaCl for 2 hours. The anti-EGFR mAb Pan was also conjugated to DM1-SMCC to form Pan-DM1. In addition, anti-TNF DM1 conjugate was prepared as control. DAR was determined by UV-VIS absorbance at 280 and 252 nm and calculated according to US 2010/0092495 A1. Drug distribution was then performed by incubating 100 µg of PanP-DM1 with 7.14 µg endo-β-N-acetylglucosaminidase F2 (Endo F2) (Zhangjiang Biotech, Inc.) at 37°C followed by the addition of Biopharmalyx Biotech, Inc.) at 37°C for 4 h. Approximately 2 µg of deglycosylated PanP-DM1 was loaded onto a Waters C4 column (2.1 x 50 mm, 1.7 µm) and eluted with a mobile phase of 25–35% aqueous acetonitrile gradient containing 0.1% FA in 6min at 60°C with a flow rate of 0.4ml/min. Finally, Biopharmalyx was used to analyze the obtained mass spectrum.

**RP-UPLC/ESI-MS analysis of deglycosylated PanP-DM1**

Samples were analyzed using reverse-phase chromatography coupled online with ESI-TOF-MS. Firstly, deglycosylation was performed by incubating 100 µg of PanP-DM1 with 7.14 µg endo-β-N-acetylglucosaminidase F2 (Endo F2) (Zhangjiang Biotech, Inc.) at 37°C for 4 h. Approximately 2 µg of deglycosylated PanP-DM1 was loaded onto a Waters C4 column (2.1 x 50 mm, 1.7 µm) and eluted with a mobile phase of 25–35% aqueous acetonitrile gradient containing 0.1% FA in 6 min at 60°C with a flow rate of 0.4ml/min. Finally, Biopharmalyx was used to analyze the obtained mass spectrum.

**EGFR binding measurements by ELISA and Flow Cytometry**

The assay was performed as described previously. Briefly, 96-well plates (Nunc) were coated with EGFR-Fc and blocked with phosphate-buffered saline (PBS) containing 10% nonfat dried milk. Indicated concentrations of antibodies were added to the plates and incubated for 1 hour at room temperature. A wash step, bound antibody was detected with horseradish peroxidase (HRP)—conjugated anti-human F(ab’)2 (Sigma) for 30 min at 37°C followed by the addition of 3,3’,5,5’-tetramethylbenzidine (TMB) substrate reagent. Absorbance was read at 450 nm. Data were fitted using a sigmoidal 4-parameter curve.

For flow cytometric analysis, A431 cells overexpressed EGFR were incubated on ice with indicated agents in fluorescence activated cell sorting (FACS) buffer (PBS buffer containing 1% fetal bovine serum) for 1 hour. Cells were washed with FACS buffer and incubated with FITC-labeled goat anti-human IgG (H’+L) secondary antibody (Life Technologies) on ice for 30 min. Then all samples were washed using FACS buffer, followed by FACS analysis using a BD FACS Calibur system (BD Biosciences).

**In vitro cytotoxicity assays**

The effects of PanP and PanP-DM1 conjugate on tumor cell viability were assessed using CCK-8 kit (Dojindo, Japan). Cells in medium containing 1% fetal bovine serum were plated in 96-well culture plates (5,000 per well for BT-474, MCF-7 and DiFi; 3,000 cells per well for H292; 2,000 cells per well for A431) and allowed to adhere overnight at 37°C. The next day, the indicated concentrations of activated-PanP, activated PanP-DM1, PanP-DM1 or anti-TNF-DM1 as control were added to each well and the cells were incubated for varying periods of time (48 h for A431 cells; 72 h for other cells). Then cell viability was determined by CCK-8 kit according to the manufacturer’s instructions.

The percentage of surviving cells was calculated using the following formula: [(A450 of experiment - A450 of background) / (A450 of untreated control - A450 of background)] *100.

**Cell cycle analysis**

This assay was performed according to previous report. Cells (1 × 10⁶/ml) were incubated with activated PanP (1 µg/ml), activated PanP-DM1 (1 µg/ml) or PanP-DM1 (1 µg/ml) for 3 h at 37°C, washed, and replaced with drug-free medium for an additional 18 h at 37°C. Cells were then fixed with 1 mL of 70% ethanol, and DNA content was determined after staining with propidium iodide by flow cytometry. Flow cytometric data were analyzed using FlowJo 7.6 software.

**Internalization determination of PanP-DM1**

This assay was conducted as previously described. A431 cells were treated with the saturating concentration of activated PanP-DM1 conjugate at 4°C for 30 minutes. Unbound conjugate was removed by washing cells. Cells were then incubated at either 4°C or 37°C. At the indicated time points, the cell surface-bound PDC was detected by flow cytometry with goat anti-human IgG FITC.

**In vivo efficacy study**

Mice were injected subcutaneously with 0.2 mL of 1 × 10⁶ A431 cells or 1 × 10⁶ NCI-H292 cells suspended in PBS into the right flank. All mice were randomly divided into groups of 10 mice each when tumor volumes reached an average of about 100 mm³ (for A431 cells) or 130 mm³ (for H292 cells). In the 2 models mentioned above, PanP (35 or 20 mg/kg), PanP-DM1 conjugate (35 or 20 mg/kg), or human IgG as control (35 or 20 mg/kg) was given i.p. (one or 2 injections as indicated). Tumor size, calculated as length × width² × 0.5. Mice were euthanized with CO₂ asphyxiation. All experiments were line DiFi was obtained from MD Anderson Cancer Center. The anti-EGFR mAb Pan and PanP were produced by State Key Laboratory of Antibody Medicine and Targeted Therapy (Shanghai, China). Recombinant human uPA was purchased from Sino Biological Inc. DM1-SMCC was purchased from Levena Biopharma Co, Ltd (China). Six-week-old female BALB/c nude mice and BALB/c mice were obtained from the Shanghai Experimental Animal Center of Chinese Academy of Sciences (Shanghai, China). All animals were treated in accordance with guidelines of the Committee on Animals of the Second Military Medical University.
conducted in accordance with institutional guidelines and under an Institutional Animal Care and Use Committee protocol.

**In vivo toxicity study**

Antigen-independent toxicity of the PDC was determined in BALB/c mice (5 mice per dose) by a single i.p. injection of PanP-DM1 at 0, 25, 50, 75 or 100 mg/kg body weight. In addition, toxicity associated with therapeutic dose was evaluated in nude mice bearing A431 and NCI-H292 cells (10 mice per group). Mice were observed for 15–20 d. Toxicity was evaluated by observing mouse body weight loss.

**Statistical analysis**

GraphPad Prism 5 Software was used for statistic calculations. Data are showed as mean ± SEM. Statistical analysis was conducted by Student’s 2 sided unpaired t-test to identify significant differences unless otherwise indicated. Differences were considered significant at P < 0.05 (*). Nonlinear regression analyses were used to fit curves.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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