Activated platelets in the tumor microenvironment for targeting of antibody-drug conjugates to tumors and metastases

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Abstract

Rationale: Platelets are increasingly recognized as mediators of tumor growth and metastasis. Hypothesizing that activated platelets in the tumor microenvironment provide a targeting epitope for tumor-directed chemotherapy, we developed an antibody-drug conjugate (ADC), comprised of a single-chain antibody (scFv) against the platelet integrin GPIIb/IIIa (scFvGPIIb/IIIa) linked to the potent chemotherapeutic microtubule inhibitor, monomethyl auristatin E (MMAE).

Methods: We developed an ADC comprised of three components: 1) A scFv which specifically binds to the high affinity, activated integrin GPIIb/IIIa on activated platelets. 2) A highly potent microtubule inhibitor, monomethyl auristatin E. 3) A drug activation/release mechanism using a linker cleavable by cathepsin B, which we demonstrate to be abundant in the tumor microenvironment. The scFvGPIIb/IIIa-MMAE was first conjugated with Cyanine7 for in vivo imaging. The therapeutic efficacy of the scFvGPIIb/IIIa-MMAE was then tested in a mouse metastasis model of triple negative breast cancer.

Results: In vitro studies confirmed that this ADC specifically binds to activated GPIIb/IIIa, and cathepsin B-mediated drug release/activation resulted in tumor cytotoxicity. In vivo fluorescence imaging demonstrated that the newly generated ADC localized to primary tumors and metastases in a mouse xenograft model of triple negative breast cancer, a difficult to treat tumor for which a selective tumor-targeting therapy remains to be clinically established. Importantly, we demonstrated that the scFvGPIIb/IIIa-MMAE displays marked efficacy as an anti-cancer agent, reducing tumor growth and preventing metastatic disease, without any discernible toxic effects.

Conclusion: Here, we demonstrate the utility of a novel ADC that targets a potent cytotoxic drug to activated platelets and specifically releases the cytotoxic agent within the confines of the tumor. This unique targeting mechanism, specific to the tumor microenvironment, holds promise as a novel therapeutic approach for the treatment of a broad range of primary tumors and metastatic disease, particularly for tumors that lack specific molecular epitopes for drug targeting.

Key words: Cancer, Activated platelets, GPIIb/IIIa, Antibody-drug conjugate
Introduction

The mainstay of treatment for many cancers remains systemic chemotherapy. However, this approach is associated with inevitable ‘off target’ effects of chemotherapy, resulting in systemic toxicity due to the limited selectivity for tumor cells. Recently, antibody-directed chemotherapy has offered a very promising approach to overcome these inherent limitations of systemic chemotherapy by leveraging the targeting capability of antibodies towards tumor-specific antigens or proteins for the delivery of chemotherapy [1]. Consequently, antibody-drug conjugates (ADC) provide the means to deliver high local concentrations of chemotherapy specifically to the tumor whilst avoiding systemic toxicity [2]. The efficacy of ADCs has been demonstrated by the success of trastuzumab emtansine for HER-2 positive breast cancer and brentuximab vedotin in anaplastic large cell lymphoma and Hodgkin lymphoma [3,4], and holds promise in malignancies with limited prognosis such as glioblastoma [5].

The efficacy of ADCs is highly contingent upon the expression and abundance of a specific target epitope on the cancer cells. However, this represents a major limitation for the use of ADCs since in several cancer types, such as triple negative breast cancer, specific molecular targets are lacking or are yet to be determined [6,7]. Here, we develop a unique strategy to overcome this limitation by targeting a specific component of the tumor microenvironment, as opposed to a molecular epitope unique to a specific cancer cell. There is growing evidence demonstrating an intimate relationship between platelets and cancer, and the abundance of activated platelets in the microenvironment of a range of tumors [8–10] including ovarian cancer [11], colorectal cancer [12] and oesophageal cancer [13]. In this regard, platelets express an abundant and specific surface epitope, the integrin glycoprotein (GP)IIb/IIIa (αMβ3; CD41/CD61). Importantly, on circulating platelets this integrin exhibits an ‘unactivated’ or low affinity conformation. In contrast, upon platelet activation, GPIIb/IIIa undergoes a confirmation change such that it adopts a high affinity conformation for ligand binding [14]. Indeed, we recently provided proof of concept that the high affinity conformation of GPIIb/IIIa on activated platelets can serve as a molecular targeting epitope for the tumor microenvironment in several types of cancers in mice and humans [15]. Therefore, targeting activated platelets in the tumor microenvironment, using a highly abundant and specific epitope, represents a novel approach for the targeted delivery of tumor directed chemotherapy.

Here, we describe the generation of a unique ADC, scFvGPIIb/IIIa-MMAE, that targets platelets in the microenvironment of tumors as a novel approach for cancer treatment. This ADC is based on three major components: 1) The targeting scFv (scFvGPIIb/IIIa), which selectively binds to an epitope that is cryptic on the low-affinity GPIIb/IIIa on circulating platelets but becomes exposed on the high affinity GPIIb/IIIa, expressed on activated platelets [16,17]. 2) A synthetic microtubule inhibitor, monomethyl auristatin E (MMAE), which is an ideal drug for targeting by utilizing its high cytotoxic potency and obviating its otherwise dominant non-specific cell toxicity. 3) A cancer-specific, Valine-citrulline (Val-Cit) linker, utilizing the abundance of cathepsin B in the tumor microenvironment for cleavage and release of the active MMAE, thereby locally releasing cytotoxic effects [18,19].

To demonstrate the functionality of our novel anti-cancer ADC, with its unique activated platelet targeting, we first confirmed the efficacy of scFvGPIIb/IIIa-MMAE for tumor killing in human cell lines of triple negative breast cancer, colorectal cancer, fibrosarcoma and prostate cancer. Next, we used a triple negative breast cancer metastasis model for proof of concept in vivo studies. Treatment of mice with scFvGPIIb/IIIa-MMAE resulted in significant regression of primary tumors and prevented metastasis without systemic side effects. Together, these findings indicate the generation of a promising ADC and establishes a unique concept that holds promise as a novel, potentially broadly applicable anti-cancer therapy, which is of relevance for both difficult to treat tumors and those with limited specific target epitopes.

Materials and Methods

Study Approval

All animal studies were conducted in strict accordance with protocols approved by the Alfred Medical Research Education Precinct Animal Ethics Committee and the Monash University Animal Ethics Committee.

Generation of targeting scFv-LPETG and coupling enzyme sortase A

The generation of the scFvGPIIb/IIIa and a control, non-binding, scFv (scFvmut) has been described previously [20]. Using polymerase chain reaction, an LPETG-tag (sortase A recognition sequence), a V5-tag and a His-tag were introduced to the C-terminal end of the scFv [21]. The entire scFv was then subcloned into a pSectag 2A vector (Invitrogen, Carlsbad, CA, United States) for expression in human
embryonic kidney (HEK) cells (Invitrogen, Carlsbad, CA, United States) [22]. Sortase A was used to induce an enzymatic reaction for the conjugation of the scFv, carrying an LPETG sequence to MMAE which was produced incorporating a triple glycine sequence. Sortase A, a transpeptidase cloned from Staphylococcus aureus was produced and purified as previously described [23]. All proteins (scFvs and sortase A) contain a 6x His-tag, which was used for purification with nickel-based affinity chromatography (Invitrogen, Carlsbad, CA, United States).

**Conjugation of scFv with MMAE and Cy7**

MMAE, carrying a Val-Cit linker and a triple glycine sequence (GGG-Val-Cit-PAB-MMAE) was synthesized by Levena Biopharma (San Diego, CA, United States). The scFvGPIIb/IIIa and scFvmut (each constructed with a LPETG-tag) were linked to GGG-Val-Cit-PAB-MMAE using a sortase A enzyme-based protocol to produce scFvGPIIb/IIIa-MMAE and scFvmut-MMAE, as described previously [21]. Excess scFv which contains a His-tag was removed using metal affinity chromatography (Invitrogen, Carlsbad, CA, United States) and excess MMAE was removed using a 10 kDa spin column. For imaging studies, Cy7 was incorporated into the conjugate by incubating scFvGPIIb/IIIa-MMAE and scFvmut-MMAE with 2x excess Cy7 via amine labeling (AAT Bioquest, Sunnyvale, CA, United States). Excess free dye was removed by dialysis in PBS. The purified scFv-Cy7-MMAE was analyzed by SDS-PAGE gel and the protein and near-infrared signal from the band of interest was confirmed using the Odyssey Imager (Licor Biosciences, Lincoln, NE, United States). Additionally, Western blot was performed with rabbit anti-MMAE antibody (Levena Biopharma, San Diego, CA, United States), detected with an HRP-anti-rabbit antibody (Cell Signaling, Danvers, MA, United States) to confirm conjugation of MMAE to the scFv.

**Preparation of platelet rich plasma and flow cytometry**

Blood was collected from healthy volunteers in citrate and centrifuged at 180 g for 10 minutes. The platelet rich plasma was then collected, stored at 37°C and used within two hours. For flow cytometry, platelet rich plasma was diluted 1:20 in Tyrode’s buffer. To induce platelet activation, ADP was added at a final concentration of 20 μM for 5 minutes before adding the scFv. Binding was determined by anti-V5-FITC (ThermoFisher Scientific, Waltham, MA, United States) or rabbit anti-MMAE antibody (Levena Biopharma, San Diego, CA, United States) and detected with an anti-rabbit mAb coupled to AF647 (Invitrogen, Carlsbad, CA, United States). Flow cytometry was performed using a FACS Fortessa scanner (BD Biosciences, Franklin Lakes, NJ, United States). Results were analyzed using the Flowlogic software.

To determine the ability of cancer cells to activate platelets, platelet rich plasma was incubated with the cancer cell lines MDA-MB-231, HT29, HT1080 and PC3 for 6 hours at 37°C. As a positive control with the same experimental setting, ADP-activated platelet rich plasma was incubated with the cancer cells for 6 hours at 37°C. The cancer cell and platelet (resting/ADP-activated platelets) mixtures were then stained with an anti-CD41-PE monoclonal antibody (BD Biosciences, Franklin Lakes, NJ, United States) and scFvGPIIb/IIIa or scFvmut binding was detected by an anti-V5-FITC monoclonal antibody (Abcam, Cambridge, United Kingdom). Flow cytometry and analysis was performed as described above.

**Cancer cell lines**

A metastatic variant of the MDA-MB-231 triple-negative breast adenocarcinoma cell line (a kind gift from Dr Zhou Ou, Fudan University Shanghai Cancer Center, China) was transduced with a lentiviral vector containing codon-optimized firefly luciferase-mCherry under the control of the ubiquitin-C promoter [24,25] and was cultured in DMEM medium + Glutamax (Life Technologies, Carlsbad, CA, United States), supplemented with 10% (v/v) FBS (Invitrogen, Carlsbad, CA, United States), at 37°C in a 5% CO2 humidified atmosphere. Human cancer cell lines HT29, HT1080 and PC3 were cultured in RPMI media (Life Technologies, Carlsbad, CA, United States) supplemented with 10% (v/v) FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin at 37°C in a 5% CO2 humidified atmosphere. Cell identity was confirmed by karyotyping.

**Cytotoxicity assays**

The cytotoxic activity of GGG-Val-Cit-PAB-MMAE and scFv-MMAE were assessed in a cytotoxicity assay in the presence of cathepsin B. To induce cathepsin B cleavage, MMAE, GGG-Val-Cit-PAB-MMAE or scFv-MMAE were incubated with 0.01 units of pre-activated cathepsin B (Sigma Aldrich, St Louis, MO, United States) for 4 hours at 37°C in 25 mM acetate buffer pH 4.8. MDA-MB-231, HT29, HT1080 and PC3 cells were seeded on a 96 well plate at 6000 cells/well overnight. The next day, cells were treated with MMAE, GGG-Val-Cit-PAB-MMAE or scFv-MMAE in the presence or absence of cathepsin B and incubated for 72 hours. Metabolism of the yellow tetrazolium salt (XTT) was determined according to the manufacturer’s instructions (Sigma Aldrich, St Louis, MO, United States). The cytotoxic activity was confirmed by karyotyping.
Louis, MO, United States). Percentage killing relative to untreated control cultures was calculated using the following formula: 100 - [(test value/untreated value) \times 100]. Each assay was repeated in triplicate.

For platelet assays, 100 µL of platelet rich plasma (containing approximately 2x10^7 platelets) was activated with 20 μM ADP. Activated platelets were incubated with scFv-MMAE for 30 minutes. To remove unbound scFv-MMAE, the platelet-scFv-MMAE mixture was centrifuged at 2000 g for 2 minutes, the supernatant was removed, and platelets were resuspended in PBS. As a control, un Washed platelet-scFv-MMAE mixture was used. Either washed or unwashed (control) platelet-scFv-MMAE mixture was added to MDA-MB-231, HT29, HT1080 and PC3 cells in the absence of exogenous cathepsin B and incubated for 72 hours before determination of XTT metabolism as described above.

**Immunohistochemical analysis of cathepsin B in tumors**

Tumors were fixed in formalin solution (Sigma Aldrich, St Louis, MO, United States) for 24 hours, paraffin embedded, and microtome sectioned (Leica, Wetzlar, Germany) to 5 µm – 30 µm onto a glass slide. Sections were deparaffinized and underwent antigen retrieval with 0.01 M Citric Acid in 90°C for 20 minutes. Tumor sections were stained overnight with an anti-cathepsin B antibody (Abcam, Cambridge, United Kingdom) and detected with an Alexa Fluor 488 labeled anti-mouse antibody (Life Technologies, Carlsbad, CA, United States) and cell surface membrane-reactive anti-sodium/potassium ATPase antibody (Abcam, Cambridge, United Kingdom) and counterstained with Hoechst® (ThermoFisher Scientific, Waltham, MA, United States) and visualized using the A1r Plus Confocal Microscope (Nikon, Shinagawa, Tokyo, Japan), 20x water objective.

**Breast cancer metastasis model**

To establish metastasis, 2 x 10^5 MDA-MB-231 cells in 20 µL PBS were injected into the fourth left mammary fat pad of 5-6 weeks old anaesthetized BALB/C athymic nude mice. Primary tumor growth was measured by caliper and tumor volume was determined using the formula \((\text{length} \times \text{width}^2) / 2\). Additionally, primary tumor growth was measured via bioluminescence using the IVIS Lumina II (Perkin Elmer, Waltham, MA, United States) imaging system by measuring luciferase activity after a tail-vein injection of 150 mg/kg D-luciferin (ThermoFisher Scientific, Waltham, MA, United States). Metastasis development was monitored via bioluminescence using IVIS Lumina II by measuring luciferase activity in the chest region, distant from the primary tumor, for a longer time point (60 sec) as previously described [25]. Metastasis development was quantified using the Living Image software v4.5.1 (Perkin Elmer, Waltham, MA, United States) by quantifying photon/s in a region of interest around the chest bioluminescence signal.

For therapy studies, mice underwent bioluminescence imaging on day 3 and day 7 post tumor inoculation to confirm tumor growth. Mice with tumors were then randomly assigned to three groups and treated with either scFvGPIIb/IIIa-MMAE, scFvmut-MMAE or left untreated. Treatment was initiated 7 days following tumor inoculation, via intravenous injection of 6 mg/kg body weight of either scFvGPIIb/IIIa-MMAE or scFvmut-MMAE followed by three additional treatments every fourth day. Primary tumor size and metastasis development was monitored twice a week as described above.

**In vivo fluorescence imaging of scFvGPIIb/IIIa-Cy7-MMAE**

Animals were injected intravenously with 20 µg of scFvGPIIb/IIIa-Cy7-MMAE or scFvmut-Cy7-MMAE. Fluorescence imaging of the ADC in mice was performed 24 hours later using the IVIS Lumina using the following settings (Filter Passband = Excitation 710-760 nm, Emission 810-875 nm). Following imaging, mice were sacrificed, organs perfused to remove circulating blood and reimaged.

**Flow cytometry and ex vivo fluorescence imaging of scFvGPIIb/IIIa-GFP and GPIIbβ**

BALB/C nude mice with MDA-MB-231 mammary tumor, or non-tumor bearing mice were injected intravenously with 20 µg of scFvGPIIb/IIIa-GFP or scFvmut-GFP and 20 µL of DyLight 649 anti-GPIIbβ (Emfret Analytics, Eibelstadt, Bayern, Germany). After 24 hours, animals were sacrificed, and tumor, spleen and femurs were extracted.

Tumor samples were fixed in formalin (Sigma Aldrich, St Louis, MO, United States) for 24 hours, paraffin embedded, and microtome sectioned (Leica, Wetzlar, Germany) to 20 µm onto a glass slide. Sections were deparaffinized, stained with Hoechst® and visualized using the A1r Plus Confocal Microscope using a 60x oil objective.

For flow cytometry, spleen sections were teased apart, disaggregated and filtered through a 100 µm filter into single cell suspensions. Bone marrow cells were extracted from the femurs and filtered through a 100 µm filter into single cell suspensions. Spleen and bone marrow cells were then suspended in 1 mL red cell lysis buffer (0.155 M NaCl, 0.01 M KHCO_3, 0.01 mM EDTA) for 10 min and neutralized withwash
buffer (PBS, 2mM EDTA, 0.1% BSA). Cells were stained with mouse CD41-PE (eBioscience, Waltham, MA, United States) and flow cytometry was performed using the Fortessa scanner. Results were analyzed using the Flowlogic software.

**Mouse blood collection and toxicity measurements**

Mouse blood was collected via submandibular bleeds into EDTA-coated microtainer collection tubes (BD Biosciences, Franklin Lakes, NJ, United States) and blood counts were performed using the XS-1000i hematologic analyzer (Sysmex Corporation, Kobe, Hyogo, Japan) to determine white blood cells (WBC) and platelets counts. For liver and kidney function tests, 500 µL of mouse blood was collected in citrate. Plasma was collected following centrifugation of blood at 1000 g for 10 minutes and alkaline phosphatase (ALP), alanine aminotransferase (ALT) and urea levels were measured using the Synchron LX20PRO System (Beckman Coulter Diagnostics, High Wycombe, United Kingdom) by Monash Pathology.

**Statistical analyses**

All data are reported as mean ± SEM of at least 3 independent assays unless otherwise noted. Statistical analyses were performed using unpaired Student’s T tests for comparison of two groups. Statistical analyses were performed using one-way ANOVA, with Dunnett’s multiple comparisons test for analysis of metastasis volume and two-way ANOVA, with Tukey’s multiple comparisons test for analysis of primary tumor growth volume. A P value of < 0.05 was considered significant.

**Results**

**ScFvGPIIb/IIIa-Cy7-MMAE binds to activated platelets**

To allow the unique targeting of a potent anti-mitotic agent to activated platelets, we developed a novel antibody-drug conjugate combining several advanced biotechnological tools and methods. We utilized a unique scFv GPIIb/IIIa, which specifically targets activated platelets, combined with a sortase A based site-directed biological conjugation method to produce a novel ADC, scFvGPIIb/IIIa-MMAE incorporating the potent anti-mitotic agent (MMAE). In order to facilitate its conjugation to the scFv and enzymatic release, a MMAE linker was designed with a triglycine (GGG) sequence, a cathepsin B cleavable peptide (Val-Cit) and a para-aminobenzalcohol (PABA) self-immolative spacer (Figure 1). Each scFv has a sortase A recognition sequence, LPETG, at the C-terminus used for a site-directed, enzymatic

**Figure 1.** Structure of GGG-Val-Cit-PAB-MMAE and labeling strategy for the generation of scFvGPIIb/IIIa-Cy7-MMAE and cathepsin B drug release. (A) scFvGPIIb/IIIa was conjugated to GGG-Val-Cit-PAB-MMAE using an enzymatic sortase A reaction, (B) followed by Cy7 conjugation, via NHS labelling to produce scFvGPIIb/IIIa-Cy7-MMAE. (C) In vivo, cathepsin B cleaves the scFvGPIIb/IIIa-Cy7-MMAE at the MMAE Val-Cit linker, releasing the potent MMAE for tumor killing.
Theranostics 2019, Vol. 9, Issue 4

coupling to the glycine sequence of GGG-Val-Cit-PAB-MMAE. To enable in vivo detection, the scFv-MMAE was labeled with a Cy7 dye by NHS labeling to yield, scFv<sub>GPIIb/IIIa</sub>-Cy7-MMAE (Figure 1). Analysis of this final construct using SDS page electrophoresis and visualized using the Odyssey reader confirmed successful Cy7 labeling of the scFv which has a molecular weight of 34 kDa (Figure 1A). The scFv<sub>GPIIb/IIIa</sub> carries a hexahistidine tag downstream of the LPETG sequence, which is cleaved following sortase A reaction, resulting in a final scFv<sub>GPIIb/IIIa</sub>-Cy7-MMAE product with reduced molecular weight compared to the uncleaved scFv<sub>GPIIb/IIIa</sub>. MMAE conjugation to the scFv was analyzed via Western blotting using an anti-MMAE antibody and confirmed by the presence of a band at ~33 kDa (Figure 1B). Additionally, flow cytometry experiments demonstrated that specific binding of the scFv<sub>GPIIb/IIIa</sub>-Cy7-MMAE construct to activated platelets was maintained post conjugation (Figure 1C). The scFv<sub>GPIIb/IIIa</sub> used in this study binds to only the activated form of platelet-specific GPIIb/IIIa, importantly both in human, as well as in mouse platelets. As a negative control, we used an scFv<sub>mut</sub>, which we have previously developed with a point mutation, such that it acts as a non-binding control (Figure 1D) [16].

Cathepsin B cleaves scFv<sub>GPIIb/IIIa</sub>-MMAE and scFv<sub>mut</sub>-MMAE to its potent and cytotoxic form

Next, we assessed the cytotoxic activity of MMAE on tumor cells in culture after the drug was released from the ADC by exogenous cathepsin B and confirmed equal dose-dependent killing of the triple-negative breast adenocarcinoma cell line MDA-MB-231 by scFv<sub>GPIIb/IIIa</sub>-MMAE and scFv<sub>mut</sub>-MMAE. On MDA-MB-231 cells, the IC<sub>50</sub> of scFv<sub>GPIIb/IIIa</sub>-MMAE was 1.98x10<sup>-10</sup> M while the IC<sub>50</sub> of the scFv<sub>mut</sub>-MMAE was 1.78x10<sup>-10</sup> M (Figure 3A). These studies confirmed that our approach of utilizing a sortase A conjugation of MMAE to the scFv allowed for a controlled and equal coupling of MMAE to the scFv and the equivalent cathepsin B-mediated release of cytotoxic MMAE by scFv<sub>GPIIb/IIIa</sub> and scFv<sub>mut</sub>.

The incorporation of a cathepsin B cleavable linker enables the activation of an inactive prodrug to an active cytotoxic form by utilizing the abundance of cathepsin B in the tumor microenvironment [26]. Prior to animal studies, in vitro studies were performed to demonstrate that the scFv<sub>GPIIb/IIIa</sub>-Cy7-MMAE is released and maintains cytotoxicity upon exposure to cathepsin B. MDA-MB-231 cells were incubated with either MMAE, GGG-Val-Cit-PAB-MMAE or scFv<sub>GPIIb/IIIa</sub>-MMAE in the absence and presence of cathepsin B. The IC<sub>50</sub> killing efficacy of GGG-Val-Cit-PAB-MMAE on MDA-MB-231 tumor cells was 1.86x10<sup>-8</sup> M and the addition of cathepsin B increased the cellular killing efficacy by approximately 40-fold to 4.70x10<sup>-10</sup> M (Figure 3B), which is similar to the IC<sub>50</sub> of the unmodified MMAE. The IC<sub>50</sub> killing efficacy of scFv<sub>GPIIb/IIIa</sub>-MMAE was 2.21x10<sup>-8</sup> M and the addition of cathepsin B enhanced the killing efficacy to 1.96x10<sup>-10</sup> M (Figure 3B). We confirmed an increased cell killing by the GGG-Val-Cit-PAB-MMAE and scFv-MMAE in the...
presence of cathepsin B, indicating release of MMAE. We also observed a lower level (~10^{-8} M) of non-specific killing of cancer cells with scFv-MMAE and GGG-Val-Cit-PAB-MMAE in the absence of exogenous Cathepsin B. We expect this to be due to the secretion of small amounts of cathepsin B from the cancer cells, or release of other cathepsins and proteases that can also cleave the Val-Cit linker, as previously reported [27–29]. These findings confirm the cytotoxic potency and the efficacy of the cathepsin B dependent release and activation of the potent MMAE from its inactive form in the newly created scFvGPIIb/IIIa-MMAE.

To investigate if the therapeutic efficacy of scFvGPIIb/IIIa-MMAE is based on its specificity for activated platelets, the scFvGPIIb/IIIa-MMAE or scFv_{mut}-MMAE was incubated with ADP-stimulated platelets in vitro. Platelets were subsequently washed to remove unbound scFvGPIIb/IIIa-MMAE or scFv_{mut}-MMAE and then added to MDA-MB-231 cells (Figure 3C). Consistent with the specific binding of the targeted ADC to activated GPIIb/IIIa, incubation of scFvGPIIb/IIIa-MMAE with activated platelets resulted in increased MDA-MB-231 cell killing, with an IC50 of 5.9x10^{-9} M. In contrast, activated platelets incubated with scFv_{mut}-MMAE demonstrated no cellular killing. As a control to confirm the equal coupling of MMAE to the scFvGPIIb/IIIa and scFv_{mut}, a mixture of activated platelets and either scFvGPIIb/IIIa-MMAE or scFv_{mut}-MMAE, which did not

![Image](http://www.thno.org)
undergo washing, were added to MDA-MB-231 tumor cells (Figure 3C). No significant differences in killing were observed between the scFvGPIIb/IIIa-MMAE and scFvmut-MMAE. The IC50 killing efficacy of scFvGPIIb/IIIa-MMAE on MDA-MB-231 was 1.27x10^{-8} M and the IC50 killing efficacy of scFvmut-MMAE on MDA-MB-231 was 1.38x10^{-8} M, thereby confirming equal coupling efficacy of MMAE to both, scFvGPIIb/IIIa and scFvmut.

To examine whether our novel ADC displays efficacy against different tumor types, these experiments were repeated using a human colorectal cell line (HT29), a human fibrosarcoma cell line (HT1080) and a human prostate cancer cell line (PC3). We first demonstrated that MDA-MB-231, HT29, HT1080 and PC3 tumor cells cultured in GGG-Val-Cit PAB-MMAE and scFvGPIIb/IIIa-MMAE, in the presence of cathepsin B were able to induce cellular killing, albeit at slightly varying concentrations (Supplementary Figure 1A). Next, HT29, HT1080 and PC3 cell lines were cultured in ADP-activated platelets mixed with scFvGPIIb/IIIa-MMAE and scFvmut-MMAE (Supplementary Figure 1B). These studies confirmed that the scFvGPIIb/IIIa-MMAE bound to activated platelets and induced cell killing of the HT29, HT1080 and PC3 cell lines, thus supporting the notion that this platelet-targeted ADC is active across a broad range of tumor cell types.

In this study, we introduce a novel approach of utilizing Val-Cit-PAB-MMAE, a cathepsin B dependent prodrug, conjugated to a scFv that targets activated platelets as a component of the tumor microenvironment. Previous studies have indicated the abundance of secreted or cell surface-associated cathepsin B within tumor cells [30,31]. To confirm the suitability of the platelet-targeting approach for therapeutic application, we investigated the presence and localization of cathepsin B within the tumor via immunofluorescence imaging of tumor xenograft sections stained with a cathepsin B antibody. Immunostaining confirmed the presence of cathepsin B within MDA-MB-231 tumor cells and the tumor microenvironment (Figure 3D). To ascertain the cellular localization of cathepsin B in tumor cells, a magnified image was acquired (Figure 3E). This confirms the intracellular and pericellular location (indicated by white arrows) of cathepsin B within tumor cells.

Cancer cells induce platelet activation and can be imaged using scFvGPIIb/IIIa-Cy7-MMAE

The ability of cancer cells to activate platelets and the functionality of the scFvGPIIb/IIIa-MMAE to target activated platelets in the tumor microenvironment was first analyzed in vitro via fluorescence imaging. MDA-MB-231, HT29, HT1080 and PC3 cells were incubated with washed human platelets and stained with anti-CD41 antibody as a platelet marker and scFvGPIIb/IIIa, as a platelet activation marker. Strikingly, flow cytometry analysis of the platelet population (as defined by CD41 expression) revealed that incubation of platelets with MDA-MB-231, HT29, HT1080 or PC3 tumor cells resulted in activation of the entire platelet population, to a similar extent as ADP-activated platelets, thus confirming the ability of scFvGPIIb/IIIa to detect tumor cell-induced platelet activation (Figure 4A and 4B, red line). In contrast, scFvmut, as negative control, showed no binding to platelets incubated with cancer cells (Figure 4B, blue line). Furthermore, gating on the cancer cell population confirmed that platelets bind directly to cancer cells, to varying degrees, depending on the cell line (Figure 4A and 4C). Indeed, the percentage of MDA-MB-231, HT29, HT1080 and PC3 cells with adherent platelets were 10%, 15%, 44% and 11%, respectively (Figure 4C). To further illustrate the platelet-cancer cell interactions, we performed immunofluorescence imaging, demonstrating that platelets can bind directly to cancer cells and that cancer cells induce platelet activation, which can be detected by binding of the activation-specific scFvGPIIb/IIIa (Figure 4D).

scFvGPIIb/IIIa-Cy7-MMAE localizes to activated platelets in the tumor microenvironment and sites of metastasis

For in vivo characterization, scFvGPIIb/IIIa-MMAE and scFvmut-MMAE were conjugated to Cy7 and injected intravenously to mice with MDA-MB-231 primary tumor that had already metastasized. Fluorescence imaging of mice performed 24 hours post injection of scFv-Cy7-MMAE demonstrated enrichment of scFvGPIIb/IIIa-Cy7-MMAE at the primary tumor region, with no tumor localization of the scFvmut-Cy7-MMAE (Figure 5A). As the MDA-MB-231 cells had been previously transduced with a lentiviral vector to express luciferase, bioluminescence imaging was used to detect areas of primary tumor as shown in Figure 5B. Concordant with in vivo imaging, ex vivo analyses showed significant enrichment of scFvGPIIb/IIIa-Cy7-MMAE but not scFvmut-Cy7-MMAE in the primary tumors (Figure 5C and 5D). Bioluminescence imaging of lung and lymph node metastases was performed by covering the primary tumor site and imaging with a longer exposure time (60 sec) (Figure 5E and 5F). We further demonstrated that the scFvGPIIb/IIIa-Cy7-MMAE localized in the lungs of MDA-MB-231 tumor-bearing mice with lung metastases (Figure 5E), but not in mice without lung metastases (Figure 5F),
confirming that scFvGPIIb/IIIa-Cy7-MMAE localization was specific to activated platelets within tumors. Again, no tumor localization of the scFvmut-Cy7-MMAE was observed in the lungs in the presence or absence of metastases (Figure 5E and 5F).

scFvGPIIb/IIIa localizes to activated platelets in the tumor microenvironment but not to resting platelets in the spleen and bone marrow

To further confirm the selectivity of the targeted ADC for platelets in the tumor microenvironment in vivo, MDA-MB-231 tumor-bearing mice were injected with scFvGPIIb/IIIa-GFP or scFvmut-GFP and a Dylight 649 anti-GP1bβ antibody. This in vivo approach for immunofluorescence was used as the activated form of GPIIb/IIIa undergoes a conformational change and antigen masking upon tissue fixation and is not recognized by the scFvGPIIb/IIIa. After 24 hours, tumors were extracted, sectioned and imaged using fluorescence microscopy to demonstrate the localization of platelets, detected by GP1bβ staining (red), confirming that platelets can bind directly to cancer cells (all n=3). (D) Fluorescence imaging (20x) demonstrated that MDA-MB-231 cells bind directly to platelets (red) and induces platelet activation as shown by positive scFvGPIIb/IIIa staining (green) and negative scFvmut staining.
Figure 5. Activated platelets are present in the tumor microenvironment and can be detected and imaged using the scFvGPIIb/IIIa. (A) In vivo fluorescence imaging of MDA-MB-231 tumor-bearing mice, injected with scFvGPIIb/IIIa-Cy7-MMAE and scFvmut-Cy7-MMAE. (B) In vivo bioluminescence imaging of MDA-MB-231 tumor-bearing mice confirming primary tumor localization in the mammary gland. (C) Ex vivo biodistribution of the primary tumor of MDA-MB-231 tumor-bearing mice injected with scFvGPIIb/IIIa-Cy7-MMAE and scFvmut-Cy7-MMAE. (D) Quantification of fluorescence signal of MDA-MB-231 tumor-bearing mice, injected with scFvGPIIb/IIIa-Cy7-MMAE and scFvmut-Cy7-MMAE (all n=3). Data expressed as mean ± SEM. *P<0.05 analyzed by unpaired Student’s T tests. (E) Representative bioluminescence in vivo lung imaging of MDA-MB-231 tumor-bearing mice demonstrated the presence of lung metastases (left panel) and representative ex vivo fluorescence imaging of the lung (right panel) demonstrating positive scFvGPIIb/IIIa-Cy7-MMAE signal but negative scFvmut-Cy7-MMAE. (F) Representative in vivo lung imaging of MDA-MB-231 tumor-bearing mice with no lung metastasis (left panel) and representative ex vivo fluorescence imaging, demonstrating negative scFvGPIIb/IIIa-Cy7-MMAE and scFvmut-Cy7-MMAE signal (right panel). BLI-Bioluminescence.

To demonstrate the specificity of the scFvGPIIb/IIIa to activated platelets in the tumor microenvironment, we next investigated whether the scFv localized to the spleen and bone marrow, which are two abundant sources of non-activated platelets. BALB/C nude mice were injected with scFvGPIIb/IIIa-GFP or scFvmut-GFP and the Dylight 649 anti-GPIbβ antibody and the femurs and spleen were extracted after 24 hours and stained for CD41. Single cell analysis via flow cytometry of the spleen and bone marrow, gated on CD41-positive cells displayed binding of the GPIbβ antibody in the spleen and bone marrow but no scFvGPIIb/IIIa binding to these regions (Figure 6C and 6D), thus confirming the specificity of scFvGPIIb/IIIa for activated platelets in vivo.

**Treatment with scFvGPIIb/IIIa-Cy7-MMAE reduces tumor growth and metastasis**

To investigate the in vivo effect of the scFvGPIIb/IIIa-MMAE therapeutic, MDA-MB-231 metastatic tumor-bearing mice were imaged for bioluminescence signal at day 3 post MDA-MB-231 inoculation, and again at day 7 to confirm the presence of growing tumors (Figure 7A). Mice with established tumors were then divided into three groups and treated with scFvGPIIb/IIIa-MMAE or scFvmut-MMAE, or left untreated, and the effect of therapy on tumor growth and metastasis formation was assessed. Strikingly, treatment of tumor-bearing mice with scFvGPIIb/IIIa-MMAE at a dose of 6 mg/kg (administered every 4 days) resulted in a marked reduction in the primary tumor size by over 70% as compared to mice treated with scFvmut-MMAE or mice in the untreated group (Figure 7B). Accordingly, a significant reduction in the development of lung and lymph node metastases on day 23, as measured by total bioluminescence, was seen in scFvGPIIb/IIIa-MMAE treated mice compared to non-targeted and vehicle controls (Figure 7C). Significantly, treatment with scFvGPIIb/IIIa-MMAE prevented the development of metastasis in 40% of mice (3 out of 7 mice), as indicated by bioluminescence imaging, while all mice in the untreated group or treated with scFvmut-MMAE developed metastases. Importantly, no therapeutic effects were observed with scFvmut-MMAE, thus highlighting the impressive ability of our targeting approach to concentrate therapeutic doses of MMAE specifically within the tumor and to prevent metastases. Consistent with our data demonstrating...
the high degree of specificity of scFv GPIIb/IIIa-MMAE for activated platelets, we did not observe any off-target side effects in treated mice. Mice were healthy during the course of the treatment, did not lose weight (Supplementary Figure 2A), and hematological parameters, liver function and renal function were within normal ranges (Supplementary Figure 2B). Taken together, these findings demonstrate the potential of therapeutic targeting of activated platelets as a strategy for the delivery of chemotherapy to maximize potency and potentially minimize systemic side effects.

Discussion

In this study we demonstrate the utility of targeting activated platelets within the tumor microenvironment as a novel strategy for the treatment of primary tumors and prevention of metastatic disease. This approach is based on the development of a unique ADC, which targets tumor-associated platelets using the activated GPIIb/IIIa as an epitope for localized delivery of MMAE, a potent synthetic anti-mitotic agent. Importantly, this novel approach allows for the controlled-release of MMAE, via tumor-derived cathepsin B, sparing the undesirable systemic side effects commonly seen with traditional chemotherapy.

The efficacy of an ADC is highly contingent upon the presence and abundance of a specific molecular target on tumor cells. This is a major limitation for the use of ADCs, as many cancer types do not express a clinically validated antigenic molecular target. In this regard, a particular advantage of our approach is the possible ubiquitous nature of activated platelets in a broad range of...
human tumors, including breast [32], colorectal [33], lung [34], ovarian [11] and esophageal [13] tumors, indicating that our platelet-targeting strategy holds promise for a large range of tumors.

Additionally, we show the ability to target the tumor stroma as a novel approach to deliver a pro-drug for the killing of tumor cells. Valine-citrulline (Val-Cit), a cathepsin B cleavable linker requires the presence of cathepsin B for cleavage and release of free MMAE, the active anti-cancer agent. The protease cathepsin B is tightly regulated in healthy tissues. However, it is well established that cathepsin B is overexpressed in tumor cells [30,31]. Moreover, tumor cells, as well as cells in the tumor microenvironment, such as stromal fibroblasts and macrophages, can secrete cathepsin B, resulting in the accumulation of cathepsin B within the tumor microenvironment [31,35–37].

There remains some debate regarding the role of cathepsin B in regulating tumor biology given it has been demonstrated to facilitate tumor progression and metastasis [38], and promote tumor apoptosis [39,40]. Nevertheless, it is well established that cathepsin B is highly expressed in the tumor microenvironment [30,31,38]. Therefore, incorporating a cathepsin B cleavable linker, which allows for the specific release of the potent cytotoxic MMAE at the tumor site, represents a major advantage of our ADC design.

Therapeutic approaches using ADCs, such as MMAE are commonly used to target antigens expressed on cancer cells. The binding of the ADC to the tumor cell is typically followed by internalization of the ADC and release of the cytotoxic drug [38,41]. However, studies by Dal Corso et al, have also demonstrated the functionality of ADCs with cathepsin B-cleavable linkers, which are coupled to an antibody with a specificity to non-internalizing targets in the tumor microenvironment [42,43]. Our study further illustrates that tumor-specific cytotoxicity can be achieved by targeting a platelet surface epitope using scFvGPIIb/IIIa-MMAE, conjugated via a Val-Cit linker. A similarly elegant and successful targeting approach for MMAE to the tumor microenvironment was recently reported by Li F et al.,

Figure 7. scFvGPIIb/IIIa-MMAE treatment inhibits tumor growth and metastasis development in a murine model of triple negative breast cancer. (A) Bioluminescence imaging and signal quantification of MDA-MB-231 tumor-bearing mice on day 3 and the same mouse imaged again at day 7 post tumor inoculation (indicated by a red arrow), which were then randomly assigned to scFvmut-MMAE, scFvGPIIb/IIIa-MMAE or untreated groups. (B) Primary tumor volume of MDA-MB-231 tumor-bearing mice treated with 6 mg/kg of scFvGPIIb/IIIa-MMAE (●) (n=7), scFvmut-MMAE (●) (n=6) or untreated (▲) (n=6). Data expressed as mean ± SEM. *P<0.05 analyzing by one-way ANOVA with Dunnett’s multiple comparison’s test. (C) Metastasis burden of MDA-MB-231 tumor-bearing mice treated with 6 mg/kg of scFvGPIIb/IIIa-MMAE (●) (n=7), scFvmut-MMAE (●) (n=6) or untreated (▲) (n=6), measured via bioluminescence imaging, 60 sec exposure, and representative images of lung and lymph node metastasis from each group. Data expressed as mean ± SEM. *P<0.05 analyzing by one-way ANOVA with Dunnett’s multiple comparison’s test. BLI -Bioluminescence.
who used the antigen-unspecific Fc-FcyR interaction to deliver a MMAE-conjugated ADC to tumor-associated macrophages, also resulting in the release of MMAE in the tumor environment [44]. Our approach demonstrates an alternative mechanism for targeting the tumor microenvironment as the scFv does not possess an Fc region. Nevertheless, both selective deliveries of MMAE to the tumor microenvironment represent novel promising anti-cancer approaches.

Previous studies have confirmed that cathepsin B can be transported to the extracellular surface (pericellular expression), leading to its secretion into the microenvironment as well as being associated with the cell surface [45,46]. This concept has recently been further supported by Kramer L et al., who demonstrated that tumors could be imaged using a cell impermeable Cathepsin B-specific DARPin labelled with Cy5.5, thus highlighting the abundance of membrane associated/secreted cathepsin B in the tumor microenvironment in vivo [47]. Furthermore, the secreted cathepsin B is made up of the “active” Cathepsin B (in contrast to the precursor form of cathepsin B, known as pro-cathepsin B) [48]. This “active” cathepsin B is required for cleavage of the Val-Cit linker, resulting in the release of the active and highly potent cytotoxic drug into the extracellular space [49,50]. Based on these findings, we postulate that scFvGPIIb/IIIa-MMAE is cleaved and activated by cathepsin B in the extracellular space. An alternative possibility is that the uncleaved ADC could be taken up by tumor cells, and the cytotoxic MMAE released by intracellular cathepsin B. The exact tumor cell killing mechanism remains to be investigated in future detailed studies.

Whilst many ADCs have utilized whole antibodies, we chose to use a scFv as an antibody format, since it offers several significant advantages. The small size allows quick and efficient penetration of the scFv based ADC to the tumor environment, whilst ensuring quick clearance of the scFv from non-affected tissues. The recombinant nature of production offers flexibility with respect to modification of the scFv and the incorporation of additional tags, such as the LPETG sequence which we have used in the sortase A conjugation method [45]. Furthermore, the scFvGPIIb/IIIa possesses two additional advantages for clinical translation. Firstly, the scFvGPIIb/IIIa was developed from a human scFv library, reducing the risk of antigenicity. Second, the scFvGPIIb/IIIa is cross reactive between mice and human GPIIb/IIIa, and therefore binds with similar affinity to activated platelets from both species [16].

Our data suggests that this newly designed ADC has the capability to target and efficiently kill a broad range of tumor cells in vitro. Therefore, future studies will be aimed at investigating the efficacy of scFvGPIIb/IIIa-MMAE across different tumor types in vivo. In addition, whilst tumors can display or acquire resistance mechanisms to MMAE, a major advantage of our scFv platform is the ability to conjugate the scFv with different cytotoxic drugs, thus providing versatility in both the range of tumors that can be targeted, and the cytotoxic payload to be delivered. Whilst our preliminary safety studies demonstrated no off-target toxicity associated with the scFvGPIIb/IIIa-MMAE, it is important to note that mice are approximately 3 to 6 times less sensitive to the toxic effects of MMAE compared to humans [46]. Thus, studies to examine for signs of systemic toxicity in other species, which share a similar MTD to humans, such as rats and cynomolgus monkeys, will be important for clinical translation. In general, further systematic toxicity studies would need to be undertaken prior to any potential clinical study.

Cancer associated thrombosis is one of the major causes of mortality in cancer patients. It is therefore common for cancer patients to be treated with anti-thrombotics [47]. As such, another important aspect for clinical translation relates to the question of whether the concurrent use of anti-thrombotic drugs impacts on the therapeutic efficacy of our ADC. Indeed, the use of GPIIb/IIIa inhibitors during anti-cancer therapy might reduce the effective binding of the GPIIb/IIIa targeting ADC. However, GPIIb/IIIa inhibitor therapy is only used, albeit infrequently, in the acute setting of percutaneous coronary intervention [14]. In addition, GPIIb/IIIa inhibitors are associated with a high bleeding risk and would frequently be contraindicated in patients with active cancer [48]. Nevertheless, cancer patients might receive treatment with aspirin or P2Y12 inhibitors. Although platelet function is partially inhibited with these drugs, they do not fully prevent the conformational change of the GPIIb/IIIa receptor [49], which exposes the targeting epitope of our ADC. Furthermore, given the multiple mechanisms by which tumors can activate platelets, and the high abundance of GPIIb/IIIa receptor on platelets, the ADC targeting epitope should still be present in high abundance following aspirin or P2Y12 therapy. Interestingly, anticoagulants such as heparin, have been demonstrated to increase cathepsin B activity [50], which could potentiate the effect of this ADC therapy. Overall, we expect that the described activated platelet targeting therapeutic approach will maintain efficacy in the presence of anti-thrombotic drugs. However, the issue of concomitant anti-thrombotic therapies requires further
investigation during the clinical development of scFvGPIIb/IIIa-MMAE.

Moreover, the ability of the scFvGPIIb/IIIa-MMAE to act as an imaging agent to detect activated platelets within tumors is an advantage in the context of concurrent treatment modalities that could affect platelet activation, and to identify various tumor types with abundant platelets as a predictor of treatment response. Therefore, as part of developing a personalized medicine approach, patients could first be imaged with a specific imaging version of scFvGPIIb/IIIa, e.g. which is coupled to a PET/MRI contrast agent, allowing the sensitive detection of tumors and metastases. Upon confirming the presence of activated platelets within tumors, patients could then be treated with scFvGPIIb/IIIa conjugated to the cytotoxic drug. This personalized theranostic approach is a particularly attractive capability of our activated-platelet targeting strategy.

In conclusion, we introduce a novel approach for the safe and effective delivery of anti-cancer therapy, within the tumor microenvironment, by targeting activated platelets. We designed and successfully tested a novel antibody-drug conjugate incorporating a scFv targeting activated GPIIb/IIIa and an additional selective drug activation/release mechanism via cathepsin B, for the delivery of the cytotoxic agent, monomethyl auristatin E specifically to the tumor site. Addressing a major clinical need, we provide proof of concept of the therapeutic efficacy of our novel ADC in treating both primary tumor and preventing metastasis using a murine model of the difficult to treat triple negative breast cancer. Moreover, we have previously established the ability of the activated platelet-specific scFv for imaging a broad range of tumors [15] which raises the prospect of an integrated approach for cancer diagnosis and therapy, by utilizing molecular imaging as a means to detect tumor-associated platelets, and therefore predict those likely to respond to platelet-targeted therapy. Together, these findings demonstrate the broad potential of our unique activated platelet-targeting approach as a novel theranostic anti-cancer strategy, especially for cancers that do not express specific molecular epitopes suitable for therapeutically targeting.

Abbreviations

ADC: antibody-drug conjugate; ADP: adenosine diphosphate; AF: Alexa Fluor; ALP: alkaline phosphatase; ALT: alanine aminotransferase; BLI: Bioluminescence; Cy7: Cyanine 7; DARPin: Designed ankyrin repeat protein; GPIIb/IIIa: Glycoprotein IIb/IIIa; IC50: half maximal inhibitory concentration; IVIS: In vivo imaging system; MMAE: monomethyl auristatin E; Mut: Mutant; NHS: N-hydroxysuccinimide; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; scFv: single-chain antibodies; Val-Cit: Valine-citrulline; HRP: horseradish peroxidase; WBC: white blood cells and XTT: tetrazolium salt.

Supplementary Material

Supplementary figures.
http://www.thno.org/v09p1154s1.pdf

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Author contributions

MLY, JDM, XW, MZ and AW designed and performed the experiments. MLY, JDM, XW, MZ, YCC, AW, CJN, EKS and GAP analyzed the data. MLY, JDM, XW, MZ, PMH, EKS, GAP and KP wrote the manuscript. AMS, EKS, PMH, GAP and KP supervised the project. GAP and KP developed the ADC and conceived the project.

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Competing Interests

KP is an inventor on patents describing activated platelet-targeting recombinant antibodies. All other authors have declared that no competing interest exists.
References


