Potential Mechanisms for Thrombocytopenia Development with Trastuzumab Emtansine (T-DM1)

Hirdesh Uppal¹, Estelle Doudement¹, Kaushiki Mahapatra¹, Walter C. Darbonne², Daniela Bumbaca³, Ben-Quan Shen³, Xiaoyan Du³, Ola Saad⁴, Kristin Bowles⁵, Steve Olsen⁶, Gail D. Lewis Phillips³, Dylan Hartley¹, Mark X. Sliwkowski⁷, Sandhya Girish⁸, Donna Dambach⁹, and Vanitha Ramakrishnan¹⁰

Abstract

Purpose: Trastuzumab-emtansine (T-DM1) is an antibody–drug conjugate (ADC) comprising the cytotoxic agent DM1 conjugated to trastuzumab with a stable linker. Thrombocytopenia was the dose-limiting toxicity in the phase I study, and grade ≥3 thrombocytopenia occurred in up to 13% of patients receiving T-DM1 in phase III studies. We investigated the mechanism of T-DM1–induced thrombocytopenia.

Experimental Design: The effect of T-DM1 on platelet function was measured by aggregometry, and by flow cytometry to detect the markers of activation. The effect of T-DM1 on differentiation and maturation of megakaryocytes (MK) from human hematopoietic stem cells was assessed by flow cytometry and microscopy. Binding, uptake, and catabolism of T-DM1 in MKs, were assessed by various techniques including fluorescence microscopy, scintigraphy to detect T-[H³]-DM1 and ¹²⁵I-T-DM1, and mass spectrometry. The role of FcRRIα was assessed using blocking antibodies and mutant constructs of trastuzumab that do not bind FcR.

Results: T-DM1 had no direct effect on platelet activation and aggregation, but it did markedly inhibit MK differentiation via a cytotoxic effect. Inhibition occurred with DM1-containing ADCs but not with trastuzumab demonstrating a role for DM1. MKs internalized these ADCs in a HER2-independent, FcRRIα-dependent manner, resulting in intracellular release of DM1. Binding and internalization of T-DM1 diminished as MKs matured; however, prolonged exposure of mature MKs to T-DM1 resulted in a disrupted cytoskeletal structure.

Conclusions: These data support the hypothesis that T-DM1–induced thrombocytopenia is mediated in large part by DM1-induced impairment of MK differentiation, with a less pronounced effect on mature MKs. Clin Cancer Res; 21(1); 123–33. ©2014 AACR.

Introduction

One of the major limitations of systemic chemotherapy for cancer is the development of dose-limiting toxicity, caused by the exposure of nontumor cells to cytotoxic agents. Antibody–drug conjugates (ADC) are composed of a cytotoxic agent conjugated to a targeted antibody via a covalent linker (1). ADCs are designed to deliver this cytotoxic agent selectively to tumor cells, thereby minimizing systemic toxicity.

Trastuzumab-emtansine (T-DM1) is an ADC comprising the cytotoxic agent DM1 conjugated via a stable thioether linker to a targeted antibody via a covalent linker (1). ADCs are designed to deliver this cytotoxic agent selectively to tumor cells, thereby minimizing systemic toxicity.

Trastuzumab-emtansine (T-DM1) is an ADC comprising the cytotoxic agent DM1 conjugated via a stable thioether linker to the humanized human EGFR2 (HER2)-targeted monoclonal antibody trastuzumab (2). T-DM1 binds to HER2 with an affinity similar to that of unconjugated trastuzumab (3). T-DM1 is internalized, and the active catabolite lysine-N²-maleimidomethyl]-cyclohexane-1-carboxylate (MCC)-DM1 is released by lysosomal degradation (4), resulting in microtubule destabilization and subsequent inhibition of cell division and proliferation of HER2-positive cancer cells (2, 3). Like trastuzumab, T-DM1 binding to cell surface HER2 results in antitumor activities—including mediation of antibody-dependent cellular cytotoxicity, inhibition of proliferative signaling through the PI3K/Akt pathway, and inhibition of proteolytic cleavage of the extracellular domain of HER2—preventing shedding of HER2 into the circulation (3).
T-DM1 is approved for the treatment of patients with previously treated metastatic breast cancer. T-DM1 has been generally well tolerated with a lower incidence of grade $\geq 3$ adverse events compared with trastuzumab plus docetaxel (46.4% vs. 90.9%; ref. 6) and lapatinib plus capecitabine (40.8% vs. 57.0%; ref. 5). T-DM1 did, however, inhibit megakaryocyte differentiation from hematopoietic stem cells. This effect appears to be mediated by DM1, which was internalized by megakaryocytes in a HER2-independent, FcγRIIa-dependent manner. Our data support the hypothesis that impaired platelet production by megakaryocytes mediates much of the thrombocytopenia observed in clinical trials, and underscore the need to evaluate the role of Fcγ receptors in toxicities seen with antibody therapeutics on nontarget cells.

**Platelet activation**

Platelet activation was measured by detecting activated GPIIb/IIIa (i.e., PAC1 binding) and P-selectin (CD62P) expression using flow cytometry. WP (100 μL of $2 \times 10^8$ [ml]) or PRP was aliquoted into 96-well conical well plates and activated at 4°C for 10 minutes using collagen (1 to 10 μg/mL, Chronolog) or TRAP ($\sim 1$ μmol/L, Sigma-Aldrich) as a positive control, or various test antibodies. Test antibodies included trastuzumab, T-DM1, and the control nonspecific ADC SB6-DM1 (13). All three test antibodies are of IgG1 isotype. Platelets were diluted 1:1 with ice-cold Tyrodes-Hepes buffer and aliquoted (40 μL aliquots) into triplicate wells containing PE-conjugated anti-CD41 antibody to identify platelets (3 μL) or isotype control and either FITC-conjugated anti-CD62P antibody or FITC-conjugated anti-PAC1 antibody (3 μL) or respective FITC-conjugated isotype controls. Following a 1-hour incubation at 4°C, platelets were diluted and assessed by flow cytometry using a FACS Calibur (BD Biosciences). For experiments assessing the effect of the various test antibodies on agonist-induced activation, WP or PRP was preincubated with the test antibodies for 30 minutes at 4°C and then collagen or TRAP was added for an additional 10-minute incubation.

**Platelet aggregation**

WP ($500 \mu L, 2 \times 10^8/\mu L$) or PRP ($500 \mu L, \sim 2 \times 10^8/\mu L$) from normal human donors was pipetted into aggregometer tubes, and aggregation was initiated by the addition of collagen (Chrono-Log: 5 μL of 10 mg/mL solution; 10 μg/mL final concentration) or TRAP (1 μmol/L) as positive controls, or various test antibodies or compounds, and measured in a lumiaggregometer (Chrono-Log 700; Chrono-Log) with stirring (1,000±1,200 rpm) at 37°C. For experiments assessing the effect of the various test antibodies on agonist-induced activation, WP or PRP was preincubated with the test antibodies at 37°C for 30 minutes. Aggregation was initiated with collagen or TRAP and was quantified in a lumiaggregometer.

**Differentiation of human MKs**

HSCs (CD133+/CD34+) were purchased from ALLCELLS. Briefly, these HSCs were enriched from bone marrow isolates of healthy donors using positive selection for CD133 and CD34. CD133+/CD34+ cells were first expanded in cytokine-enriched hematopoietic expansion media (StemSpan CC100, Stem Cell Technologies) for 5 days, then incubated in MK differentiation media [StemSpan CC100 supplemented with thrombopoietin (100 ng/mL), SCF (25 ng/mL), IL3 (10 ng/mL), IL6 (10 ng/mL), IL11 (10 ng/mL), and IL9 (10 ng/mL)], for 14 days to obtain multinucleated MKs. Evaluation of the MK differentiation process was performed by flow cytometry, using the MK lineage-specific markers CD41 and CD61 (Supplementary Fig. S1B). To obtain mature MKs, day 14 MKs (i.e., immature MKs) were cultured in the same medium for an additional 14 to 16 days (Supplementary Fig. S1B).

**MK production and cell viability**

**Determination of IC50**

To determine the IC50 of T-DM1 on immature MKs, CD133+ /CD34+ cells were resuspended in MK differentiation medium (750,000 cells/well) and treated with different concentrations of T-DM1, trastuzumab, SB6-DM1, or vehicle (100 μg/mL, 25 μg/mL, 6.25 μg/mL, 1.56 μg/mL, 0.39 μg/mL, 0.098 μg/mL) for 14 days. At days 3, 6, 9, and 14, three aliquots (25 μL) from each well were evaluated for cellular ATP levels using CellTiter-Glo

**Materials and Methods**

**Platelet isolation**

Platelet-rich plasma (PRP) was isolated by low-speed centrifugation of whole blood from normal human donors drawn into trisodium citrate containing bivalirudin and apyrase. For washed platelets (WP), whole blood was obtained from normal human volunteers into acid citrate dextrose. WPs were prepared as described (12) by centrifugation twice in CGS buffer. Platelets (WP), whole blood was obtained from normal human donors and resuspended in Tyrodes-Hepes buffer at 2 $\times 10^8$/ml for further experiments. For activation experiments, CaCl2 (final concentration 1 mmol/L) was added, and platelets were allowed to rest for 15 minutes at room temperature before use.

**Platelet activation**

Platelet activation was measured by detecting activated GPIIb/IIIa (i.e., PAC1 binding) and P-selectin (CD62P) expression using flow cytometry. WP (100 μL of $2 \times 10^8$ [ml]) or PRP was aliquoted into 96-well conical well plates and activated at 4°C for 10 minutes using collagen (1 to 10 μg/mL, Chronolog) or TRAP ($\sim 1$ μmol/L, Sigma-Aldrich) as a positive control, or various test antibodies. Test antibodies included trastuzumab, T-DM1, and the control nonspecific ADC SB6-DM1 (13). All three test antibodies are of IgG1 isotype. Platelets were diluted 1:1 with ice-cold Tyrodes-Hepes buffer and aliquoted (40 μL aliquots) into triplicate wells containing PE-conjugated anti-CD41 antibody to identify platelets (3 μL) or isotype control and either FITC-conjugated anti-CD62P antibody or FITC-conjugated anti-PAC1 antibody (3 μL) or respective FITC-conjugated isotype controls. Following a 1-hour incubation at 4°C, platelets were diluted and assessed by flow cytometry using a FACS Calibur (BD Biosciences). For experiments assessing the effect of the various test antibodies on agonist-induced activation, WP or PRP was preincubated with the test antibodies for 30 minutes at 4°C and then collagen or TRAP was added for an additional 10-minute incubation.

**Platelet aggregation**

WP ($500 \mu L, 2 \times 10^8/\mu L$) or PRP ($500 \mu L, \sim 2 \times 10^8/\mu L$) from normal human donors was pipetted into aggregometer tubes, and aggregation was initiated by the addition of collagen (Chrono-Log: 5 μL of 10 mg/mL solution; 10 μg/mL final concentration) or TRAP (1 μmol/L) as positive controls, or various test antibodies or compounds, and measured in a lumiaggregometer (Chrono-Log 700; Chrono-Log) with stirring (1,000±1,200 rpm) at 37°C. For experiments assessing the effect of the various test antibodies on agonist-induced activation, WP or PRP was preincubated with the test antibodies at 37°C for 30 minutes. Aggregation was initiated with collagen or TRAP and was quantified in a lumiaggregometer.

**Differentiation of human MKs**

HSCs (CD133+/CD34+) were purchased from ALLCELLS. Briefly, these HSCs were enriched from bone marrow isolates of healthy donors using positive selection for CD133 and CD34. CD133+/CD34+ cells were first expanded in cytokine-enriched hematopoietic expansion media (StemSpan CC100, Stem Cell Technologies) for 5 days, then incubated in MK differentiation media [StemSpan CC100 supplemented with thrombopoietin (100 ng/mL), SCF (25 ng/mL), IL3 (10 ng/mL), IL6 (10 ng/mL), IL11 (10 ng/mL), and IL9 (10 ng/mL)], for 14 days to obtain multinucleated MKs. Evaluation of the MK differentiation process was performed by flow cytometry, using the MK lineage-specific markers CD41 and CD61 (Supplementary Fig. S1B). To obtain mature MKs, day 14 MKs (i.e., immature MKs) were cultured in the same medium for an additional 14 to 16 days (Supplementary Fig. S1B).

**MK production and cell viability**

**Determination of IC50**

To determine the IC50 of T-DM1 on immature MKs, CD133+ /CD34+ cells were resuspended in MK differentiation medium (750,000 cells/well) and treated with different concentrations of T-DM1, trastuzumab, SB6-DM1, or vehicle (100 μg/mL, 25 μg/mL, 6.25 μg/mL, 1.56 μg/mL, 0.39 μg/mL, 0.098 μg/mL) for 14 days. At days 3, 6, 9, and 14, three aliquots (25 μL) from each well were evaluated for cellular ATP levels using CellTiter-Glo
luminescence cell viability reagent (Promega). The IC₅₀ concentrations were calculated using a four-parameter logistic regression model.

For maturing MKs (day 14 cells), aliquots (100 μL) of cells treated with T-DM1, trastuzumab, or 5B6-DM1 (6.25 μg/mL) at days 17, 20, 23, and 28 postdifferentiation were assessed in the presence of antibodies (25 μM) expanded for 5 days and differentiated into MKs for 30 days in T-DM1, trastuzumab, 5B6-DM1, or vehicle (6.25 μM). MKs. Cells in MK differentiation medium were treated with lerie) were induced to differentiate for 14 days to obtain immature MKs. Cells in the sample were harvested on days 0, 9, and 14 of differentiation, the viable cells were counted with an automated cell counter (ViCell, Beckman Coulter). MK number was calculated as follows for each condition and the percentage of day 0 control was determined:

# MK cells per time point = (%CD41⁺/CD61⁺) × # of viable cells in the sample

For ploidy determination, HSCs (CD133⁺/CD34⁻) were expanded for 5 days and differentiated into MKs for 30 days in the presence of antibodies (25 μg/mL). Cells were harvested on selected days during MK differentiation and maturation, washed with cold PBS (0.5 mL), resuspended in FSB (500 μL), and analyzed by flow cytometry gated for live cells with doublet exclusion and appropriate isotype-matched controls and unstained cells as negative controls. An anti-mouse IgG Compensation Plus bead kit (BD Biosciences) was used for compensation. The MK cell number was calculated as follows for each condition and the percentage of day 0 control was determined:

# MK cells per time point = (%CD41⁺/CD61⁺) × # of viable cells in the sample

Flow-cytometric analysis.

CD133⁺/CD34⁻ cells (expanded for 5 days as described earlier) were induced to differentiate for 14 days to obtain immature MKs. Cells in MK differentiation medium were treated with T-DM1, trastuzumab, 5B6-DM1, or vehicle (6.25 μg/mL). At days 3, 6, 9, and 14 of differentiation, the viable cells were counted with an automated cell counter (ViCell, Beckman Coulter). MK number was determined as follows. An aliquot (500,000 cells per condition) was harvested and washed twice and then incubated for 30 minutes with APC-conjugated mouse anti-human CD41 (BD Pharmingen) and PE-conjugated mouse anti-human CD61 (Miltenyi Biotec Inc.) in the dark at room temperature. Cells were then washed three times with FACS Stain Buffer (FSB; BD Pharmingen) by repeated centrifugation, resuspended in FSB (500 μL), and analyzed by flow cytometry gated for live cells with doublet exclusion and appropriate isotype-matched controls and unstained cells as negative controls. An anti-mouse IgG Compensation Plus bead kit (BD Biosciences) was used for compensation. The MK cell number was calculated as follows for each condition and the percentage of day 0 control was determined:

# MK cells per time point = (%CD41⁺/CD61⁺) × # of viable cells in the sample

For ploidy determination, HSCs (CD133⁺/CD34⁻) were expanded for 5 days and differentiated into MKs for 30 days in the presence of antibodies (25 μg/mL). Cells were harvested on selected days during MK differentiation and maturation, washed with cold PBS (0.5 mL), resuspended in FSB (500 μL), and stained for 5 minutes in the dark at room temperature with Hoechst 33342 (1:10,000; Molecular Probes, Life Technologies). The cells were washed three times, resuspended in FSB (500 μL), and analyzed by flow cytometry using Cytobank Flow Cytometer Data Analysis Software (Cytobank, Inc.).

Quantification of catabolites by LC/MS-MS

Catabolite (DM1, MCC-DM1, and Lys-MCC-DM1) concentrations were determined in MK extracts prepared from days 0, 9, and 20. For analysis of MCC-DM1 and Lys-MCC-DM1, an aliquot (30 μL) was extracted by protein precipitation using 80/20 acetonitrile/water (120 μL) containing maytansine (7.5 nmol/L) as an internal standard. To measure DM1, an aliquot (30 μL) of the MK lysates was first treated at 37°C for 15 minutes at pH 6.6 with Tris (2-carboxyethyl)phosphine (1.25 μL, 26 mmol/L, ThermoScientific Pierce) to release any disulfide-bound DM1, and then extracted as described above. The free sulfhydryl on DM1 was blocked by conversion to DM1 N-ethyl maleimide (NEM; 7.9 μL, 25 mmol/L, Sigma-Aldrich) by incubation at 37°C for 45 minutes with NEM.

LC/MS-MS analysis of the extracted samples was conducted using chromatography on a C12 analytical column (Synergi MAX RP 80 A; Phenomenex), followed by Turbolon Spray ionization using an AB SCIEX QTRAP 5500 mass spectrometer. Multiple reaction monitoring scan mode was used for quantification. Transition 845.1/485.1 was monitored for DM1-NEM, 738.2/547.2 for DM1, 1103.2/485.2 for MCC-DM1, 1118.3/547.5 for Lys-MCC-DM1, and 692.3/547.4 for maytansine. The DM1-NEM standard curve had a linear range from 0.488 nmol/L to 500.0 nmol/L. The DM1 transition was monitored only to ensure the completion of the NEM derivatization reaction. The MCC-DM1 standard curve had a linear range from 1.953 nmol/L to 500.0 nmol/L and the Lys-MCC-DM1 standard curve had a linear range from 0.488 nmol/L to 500.0 nmol/L. Data were analyzed using Analyst 1.5.2 software (AB SCIEX).
media and wash supernatants were collected and used for radioactivity determination. Radioactivity associated with the cell pellet, as well as the media and washes, were quantified by γ-scintigraphy. The percent radioactivity per 10^6 cells for each fraction was determined.

Results

Effect of T-DM1 on platelet activation and aggregation

To determine whether the thrombocytopenia observed in T-DM1–treated patients was caused, at least in part, by a direct effect on mature platelets, we evaluated the effects of T-DM1 and DM1 on the function of platelets isolated from normal donors. Platelets were isolated and utilized either as PRP or WP, as described in the Materials and Methods section, and incubated with T-DM1, DM1, or controls. Neither T-DM1 nor DM1 directly induced platelet activation in PRP, as measured by the expression of activated GPIIb/IIIa (i.e., PAC1 binding; Fig. 1A) and CD62P (P-Selectin; B) expression using collagen and TRAP as positive controls. Isotype-matched controls were used as negative controls for flow cytometry. Representative data with SDs are shown. C, effects of T-DM1, trastuzumab, and a control ADC (anti-CD22-MCC-DM1) on ex vivo platelet aggregation in PRP. D, effects of various concentrations of DM1 on ex vivo platelet aggregation in PRP. All experiments were conducted in duplicate on platelets from two different donors, and representative data are shown.

Figure 1. T-DM1 does not induce activation or aggregation of platelets. Effects of T-DM1, trastuzumab, DM1, and control ADCs (anti-CD22-MCC-DM1, trastuzumab-sppDM1) on ex vivo platelet activation in PRP by flow cytometry to measure PAC1 binding (A) and CD62P (P-Selectin; B) expression using collagen and TRAP as positive controls. Isotype-matched controls were used as negative controls for flow cytometry. Representative data with SDs are shown. C, effects of T-DM1, trastuzumab, and a control ADC (anti-CD22-MCC-DM1) on ex vivo platelet aggregation in PRP. D, effects of various concentrations of DM1 on ex vivo platelet aggregation in PRP. All experiments were conducted in duplicate on platelets from two different donors, and representative data are shown.

Increased PAC1 binding and CD62P expression induced by collagen or TRAP were unaffected by pretreatment with T-DM1, trastuzumab, or control ADC (Fig. 2A and B), or by clinically relevant concentrations of DM1 (Fig. 2C and D). Similarly, neither T-DM1 (Fig. 2E) nor clinically relevant concentrations of DM1 affected agonist-induced platelet aggregation (Fig. 2F). However, at high concentrations (i.e., 100 nmol/L, which is approximately 12,500-fold higher than concentrations measured in patient samples), DM1 inhibited agonist-induced platelet aggregation by 77% (Supplementary Fig. S3).

Effect of T-DM1 on MK differentiation from HSCs

In the absence of a direct, observable effect on platelet activation or aggregation, we evaluated the potential effects of T-DM1 on generation of MKs from HSCs (i.e., CD133+/CD34+ cells) over time.

Morphology.

On day 9 of differentiation, trastuzumab had no observable effect on MK morphology compared with vehicle control (Supplementary Fig. S4A and S4B). However, treatment with the ADCs T-DM1 or 5B6-DM1 during HSC differentiation altered the cell morphology, inducing a vacuolated morphology consistent with MK death (ref. 20; Supplementary Fig. S4C and S4D, respectively).

Overall viability.

Differentiation of HSCs into MKs can be monitored via the expression of the specific MK markers CD41 and CD61. In our in vitro model, the percentage of CD41-expressing cells increases over time during differentiation, reaching...
We investigated the effect of T-DM1 on HSCs differentiating into MKs and determined the dose–response relationship of this effect (Fig. 3A). T-DM1 appeared to decrease the number of MKs by day 3 with an approximate IC_{50} of 7 μg/mL (~50 nmol/L). Decreased MK viability after T-DM1 treatment was more pronounced at day 6 with an IC_{50} of approximately 3 μg/mL (~20 nmol/L), and the IC_{50} remained stable through day 14. Trastuzumab did not decrease MK viability at any dose or time point, whereas the control ADC SB6-DM1 showed similar viability decreases as T-DM1, suggesting that the T-DM1 effect was due to its DM1 component and not its trastuzumab component.

**Viability of immature MKs.**

To further investigate the effect of T-DM1 on differentiation of HSCs into MKs, we used flow cytometry to measure total cell number, number of MKs (i.e., CD41+/CD61+ cells), and cell viability at days 3, 6, 9, and 14 of differentiation (Fig. 3B and D). The number of vehicle- and trastuzumab-treated cells increased over the 14-day time course, reaching a maximal increase of 120-fold over day 0. Continuous exposure of HSCs to DM1 conjugates resulted in a marked decrease relative to vehicle-treated and trastuzumab-treated cells in the percentage of MKs (Fig. 3B). Decreased MK production was observed as early as day 6 (~50%), and MK numbers decreased further at day 9 (~90%) and remained at this level at day 14. These changes in MK cell number correlated with a decrease in cell viability (Fig. 3D). These results suggest that decreased cell viability of HSCs differentiating into MKs induced by T-DM1 is mediated by the chemotherapeutic component of T-DM1 (i.e., DM1) and not by the antibody (i.e., trastuzumab).

**Viability of maturing MKs.**

We conducted similar experiments to determine the effect of T-DM1 on maturing MKs (i.e., days 14–28 of differentiation). By day 28, the number of control- and trastuzumab-treated MKs had increased by approximately 100% compared with day 14 control- and trastuzumab-treated cells (Fig. 3C). As early as day 20, there
was an observable reduction in MK cell number (\(-40\%\)) in the T-DM1- and 5B6-DM1-treated cells. MK numbers decreased further at day 23 (\(-75\%\)) and remained at this level at day 28 though this decrease in MK cell number was not as great as that seen in differentiating MKs. The decreases in MK cell number correlated with a decrease in cell viability (Fig. 3E).

Taken together, these data suggest that T-DM1 has a cytotoxic effect on both differentiating and maturing MKs that is mediated by its DM1 component and that the effect is more pronounced in differentiating MKs than in maturing MKs.

Effect of T-DM1 on MK ploidy

To further investigate the effect of T-DM1 on MK differentiation from HSCs, we used flow cytometry to evaluate cell ploidy over time (Fig. 4). We observed the expected progression of MK ploidy in the control HSCs over time, with a gradual shift of the cell population from largely 2N at day 4 to a broad population of 64N to 128N cells by day 30. Continuous exposure of developing MKs to DM1-containing ADCs (T-DM1 or 5B6-DM1) arrested the normal progression of megakaryocytogenesis, as evidenced by inhibition of increased nucleoploidy as early as day 10, resulting in a single broad peak of predominantly 2N to 4N cells by day 30. Unconjugated trastuzumab did not appear to affect megakaryocytogenesis as trastuzumab-treated HSCs showed a similar nucleoploidy pattern as control-treated cells.

MK uptake and catabolism of T-DM1

To determine whether the reduction in MK number and the arresting of MK development following exposure to T-DM1 were due to the uptake of T-DM1, we assessed the time course of T-[\(^{3}H\)]-DM1 uptake in HSCs differentiating into MKs. Uptake of T-DM1 was dependent on the differentiation stage of the cells, with
The greatest uptake occurring on days 0 through 6 (Supplementary Fig. S5A, protein-bound DM1). The intracellular concentrations of T-DM1 catabolites over time are shown in Table 1. These data are consistent with the viability data demonstrating that precursor MKs (i.e., differentiating MKs) are more sensitive to treatment with DM1-containing antibodies than maturing MKs. In contrast with its pathway in tumor cells (4), T-DM1 was not detectable in lysosomes, as indicated by a lack of costaining of T-DM1 and the lysosomal marker Cy3-LAMP1 (Supplementary Fig. S5B).

To determine whether the uptake of T-DM1 was mediated (completely or in part) through HER2, we first determined whether isolated MK lineage cells (day 14) express HER2. HER2 mRNA was not detected in platelets (Supplementary Fig. S6A) and was detected at only very low levels in CD61+/0 and CD61+/MKs [0.09 and 0.06, respectively, relative to the housekeeping gene HP1BP3 (mRNA levels = 1)]. In contrast, MCF-7 breast cancer cells and normal keratinocytes, which are considered to express normal levels of HER2, showed HP1BP3 mRNA expression levels of 1.08 and 1.28, respectively, compared with HP1BP3. Furthermore, Western blot analysis of HER2 expression in MK lineage cells (CD61− and CD61+ populations) and purified platelets (Supplementary Fig. S6B) demonstrated no detectable HER2 protein, indicating that T-DM1 uptake, as well as other effects of T-DM1 on these cells, is not HER2 mediated. These data suggest that the entry of DM1 conjugates into MKs does not occur through HER2 but apparently through binding to FcγRIIa. Trastuzumab, T-DM1, and 5B6-DM1 are all IgG1 isotypes.

To determine whether T-DM1 is internalized by FcγRIIa, fluorecently labeled T-DM1 and trastuzumab were incubated with MKs and evaluated over time. Both T-DM1 (Fig. 5A and C) and trastuzumab (Fig. 5B and C) showed clear binding to the surface of MKs. Flow-cytometric analysis of the surface-bound fluorescent antibodies indicated that maximal binding occurred at 4 hours (Fig. 5C). By 8 hours, the antibodies had been internalized and detection on the cell surface was significantly decreased. When MKs were preincubated with the FcγRII-blocking antibody anti-CD32, surface binding and internalization of both fluorescently labeled T-DM1 (Fig. 5A and C) and trastuzumab (Fig. 5B and C) were markedly decreased (>50%), indicating that FcγRIIa at least partially contributes to the mechanism of T-DM1 and trastuzumab binding and uptake.

To confirm that the uptake of T-DM1 into MKs is HER2 independent and at least partially mediated by FcγRIIa, we evaluated the MK uptake of trastuzumab using two different
The present study suggests that the thrombocytopenia observed in some T-DM1–treated patients is not immune mediated and may result from decreased platelet production, which is consistent with inhibition of MK differentiation, with no direct functional effect on platelets. The most pronounced effects of T-DM1 appear to occur during the differentiation process from HSCs to MKs. Our data show that trastuzumab, T-DM1, and a control DM1-containing ADC were all internalized by MKs and that HER2 was not expressed on MKs or platelets. Internalization could be inhibited by either blockade of the interaction between FcRRIa using anti-CD32 or by using the Fc-mutant trastuzumab-DANA, which is unable to bind FcRRIa. Furthermore, the observed effect on MKs occurred with T-DM1 and the control DM1-containing ADC, but not with trastuzumab. These data support the hypothesis that the effects on MK formation are mediated by the DM1 component of T-DM1 but require an interaction of the Fc domain of T-DM1 with FcRRIa for internalization. The mechanism of release of the DM1 moiety from the internalized complex is at present unknown, as we could not detect colocalization of the internalized antibody with lysosomal LAMP1 by immunofluorescence.

Our data are consistent with a previous report which found—using different methods—that T-DM1–induced platelet decreases by decreasing platelet production via an effect on the cytoskeleton of differentiating MKs (13). The proposed mechanism of decreased platelet production (i.e., cytoskeletal disruption in differentiating MKs) is also consistent with our data, showing cytoskeletal disorganization in maturing MKs when they were subjected to prolonged T-DM1 exposure (Supplementary Fig. S7B and S7D). We also show effects in earlier stages of differentiation and speculate that this occurs via a similar mechanism. Our data differ from those reported by Thon and colleagues in that these investigators concluded that T-DM1–induced thrombocytopenia occurs via a mechanism that is both HER2 and FcRRIa independent, based on the controls used and due to the fact that mouse MKs and platelets do not express FcRRIa (25). Our data clearly demonstrate an FcRRIa-dependent mechanism of internalization of T-DM1 in human MKs. Differences between the results found in the two studies could be attributed partly to differences in experimental methodologies and species differences.

T-DM1–mediated effects appeared to be more pronounced in differentiating MKs (i.e., day 0–14 cells) than in maturing MKs (day 14–28 cells). Differentiating MKs bound, internalized, and catabolized T-DM1 to a much greater degree than maturing MKs. However, T-DM1 did have an effect on maturing MKs as noted, above. T-DM1 exposure resulted in decreased cell viability (but to a lesser extent than that seen in differentiating MKs) and induced disruption of the cytoskeletal structure in these cells.

The mechanisms of T-DM1–induced thrombocytopenia described here are consistent with the pattern of cyclic decline and rebound of platelet levels seen during treatment with T-DM1 (7, 10). In most patients receiving T-DM1, platelet levels show an acute drop and reach a nadir during cycle 1 and return to baseline levels between doses (6, 7, 9, 10). Some patients do exhibit a slow drift downward in platelet counts with repeated cycles of T-DM1, resembling the pattern induced by cumulative myelosuppression seen with other cytotoxic agents (10). However, even in those patients with a slow downward shift in platelet levels over subsequent cycles, platelet counts tend to stabilize at a level less severe than grade 3 thrombocytopenia (10). Indeed, grade 3 or
higher thrombocytopenia occurred in only 4.7% to 12.9% of patients in the phase III studies with T-DM1 (6, 8). The kinetics of thrombocytopenia seen during treatment suggest that T-DM1 affects a specific platelet precursor pool. We further hypothesize that depletion of this pool results in either a shift in the equilibrium in the bone marrow to generate more platelet precursor cells to manage the platelet depletion (26) or that a new lineage that is less sensitive to T-DM1 becomes dominant and platelet counts stabilize (10), or both. This hypothesis is consistent with our in vitro studies that show a profound effect on platelet precursors (proliferating MKs).

In phase III studies of T-DM1, other severe hematologic toxicities occurred with much lower frequency than thrombocytopenia. Grade ≥3 anemia occurred in 2.7% of patients, grade ≥3 neutropenia occurred in 2.0% to 2.5%, and grade ≥3 leucopenia occurred in <2% of patients (6, 8). These clinical data are consistent with an FcγRIIa-dependent mechanism because FcγRIIa is expressed at relatively low (or absent) levels in CD34+ cells isolated from human cord blood (27) and in lymphocyte lineages in normal human bone marrow (28). However CD32 is expressed in myeloid lineages (28). It is interesting to speculate that the small fraction of patients who exhibit a slow downward drift in platelet counts over time may indeed have a polymorphism(s) in FcγRIIa or a different pattern of FcγRIIa expression which renders their MKs and/or other hematologic lineage precursors (myeloid) more susceptible to T-DM1. Additional studies to evaluate biomarkers (such as FcR polymorphisms) associated with increased susceptibility to T-DM1–induced thrombocytopenia are underway to further characterize the mechanism of T-DM1–induced thrombocytopenia.

**Figure 5.**
FcγRIIa mediates T-DM1 internalization. Fluorescently conjugated (A) T-DM1 and (B) trastuzumab are internalized by MKs (differentiated from HSCs for 14 days; panels 1–3). Preincubation with the FcγRII-blocking antibody anti-CD32 results in a marked decrease in fluorescently labeled T-DM1 (A, panels 4–6) and trastuzumab (B, panels 4–6) with greater surface localization. C, quantification of surface binding of T-DM1 and trastuzumab, as assessed by flow cytometry. D, uptake of 111In-trastuzumab, 111In-trastuzumab-DANA, 125I-trastuzumab, and 125I-trastuzumab-DANA in HSCs at day 0 of differentiation as indicated by total counts per minute. E, 125I-trastuzumab and 125I-trastuzumab-DANA in HSCs at day 3 of differentiation as indicated by total counts per minute. Error bars indicate SD.
The cyclical pattern of platelet counts over time seen in clinical studies may be explained by the interaction between T-DM1 and FcγRIIA. The affinity of human IgG1 for FcγRIIA is 0.85 to 0.90 μmol/L (29). At the established clinical dose of 3.6 mg/kg q3w, the observed Cmax of T-DM1 is 83.4 μg/mL (30). At this concentration, the occupancy of FcγRIIA would be expected to be approximately 35%. We postulate that binding of T-DM1, near or at its Cmax to FcγRIIA drives sufficient transient internalization and subsequent degradation of the conjugate to release DM1. This in turn impairs the proliferation and differentiation of proplatelet precursor(s), which temporarily depletes this species from contributing to platelet production. The transient nature of the binding, internalization, and degradation process may then explain the transient and reversible nature of the thrombocytopenia observed in patients treated with T-DM1.

Taken together, these data support the hypothesis that the thrombocytopenia observed in clinical trials with T-DM1 is mediated in large part by impaired platelet production from MKs within the bone marrow. We further hypothesize that other ADCs with an IgG1 backbone conjugated to a tubulin inhibitor via an MCC linker could potentially result in a clinical risk for dose-limiting thrombocytopenia.

Disclosure of Potential Conflicts of Interest
H. Uppal is an employee of Medivation Inc. G.D. Lewis Phillips is an employee of Genentech. M.X. Slwikowski holds ownership interest (including patients) in Roche. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: H. Uppal, K. Mahapatra, O. Saad, S. Olsen, D. Hartley, M.X. Slwikowski, S. Girish, V. Ramakrishnan
Writing, review, and/or revision of the manuscript: H. Uppal, E. Doudement, K. Mahapatra, W.C. Darbonne, D. Bumbaca, B.-Q. Shen, O. Saad, S. Olsen, G.D. Lewis Phillips, D. Hartley, M.X. Slwikowski, S. Girish, V. Ramakrishnan
Other (conduct of experiments): H. Uppal, K. Mahapatra, B.-Q. Shen, D. Hartley, V. Ramakrishnan
Other (designed and implemented the flow cytometry and imaging panels across multiple human bone marrow donors for statistical significance) to study the mechanism of thrombocytopenia upon treatment with TDM1: K. Mahapatra

Acknowledgments
The authors thank Jay Tibbitts, Jun Guo, Guangmin Li, and Neelima Koppada.

Grant Support
This work was supported by Genentech Inc. Support for third-party writing assistance, furnished by Holly Strausbaugh, was provided by Genentech, Inc.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 12, 2014; revised October 9, 2014; accepted October 13, 2014; published OnlineFirst November 4, 2014.

References


Potential Mechanisms for Thrombocytopenia Development with Trastuzumab Emtansine (T-DM1)

Hirdesh Uppal, Estelle Doudement, Kaushiki Mahapatra, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-14-2093

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2014/11/05/1078-0432.CCR-14-2093.DC1

Cited articles
This article cites 29 articles, 17 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/21/1/123.full.html#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
/content/21/1/123.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.