Ado-trastuzumab emtansine targets hepatocytes via human epidermal growth factor receptor 2 to induce hepatotoxicity

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Running title: T-DM1 induces hepatotoxicity
Keywords: ado-trastuzumab emtansine/T-DM1, hepatotoxicity, HER2, HER2-positive breast cancer, drug-induced liver injury.

Word account: Abstract (245); Text (5809); figures and table (6)

Financial Information: All authors declared no competing financial interests with industry.

This study is supported by the Interagency Oncology Task Force Joint Fellowship Program sponsored by the United States Food and Drug Administration and National Cancer Institute. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Dr. Haoheng Yan is an IOTF fellow. This work was also supported by the Food and Drug Administration Office of Women’s Health. This project was also supported in part by an appointment to the ORISE Research Participation Program at the Center for Drug Evaluation and Research, U.S. Food and Drug Administration, administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and FDA. Dr. Nishant Mohan is an ORISE research fellow supported by Food and Drug Administration Office of Women’s Health.
Abstract

Ado-trastuzumab emtansine (T-DM1) is an antibody-drug conjugate (ADC) approved for the treatment of HER2-positive metastatic breast cancer. It consists of trastuzumab, a humanized monoclonal antibody directed against human epidermal growth factor receptor 2 (HER2) and a microtubule inhibitor DM1 conjugated to trastuzumab via a thioether linker. Hepatotoxicity is one of the serious adverse events associated with T-DM1 therapy. Mechanisms underlying T-DM1-induced hepatotoxicity remain elusive. Here, we use hepatocytes and mouse as models to investigate the mechanisms of T-DM1-induced hepatotoxicity. We show that T-DM1 is internalized upon binding to cell surface HER2 and is co-localized with LAMP1, resulting in DM1-associated cytotoxicity, including disorganized microtubules, nuclear fragmentation/multiple nuclei, and cell growth inhibition. We further demonstrate that T-DM1 treatment significantly increases the serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH) in mice, and induces inflammation and necrosis in liver tissues and that T-DM1-induced hepatotoxicity is dose dependent. Moreover, the gene expression of TNFα in liver tissues is significantly increased in mice treated with T-DM1 as compared with that treated with trastuzumab or vehicle. We propose that T-DM1-induced upregulation of TNFα enhances the liver injury that may be initially caused by DM1-mediated intracellular damage. Our proposal is underscored by the fact that T-DM1 induces the outer mitochondrial membrane rupture, a typical morphological change in the mitochondrial-dependent apoptosis, and mitochondrial membrane potential dysfunction. Our work provides mechanistic insights into T-DM1-induced hepatotoxicity, which may yield novel strategies to manage liver injury induced by T-DM1 or other ADCs.
Introduction

Antibody-drug conjugates (ADCs) are an emerging group of therapeutic agents that are generated by covalent attachment of cytotoxic agents to monoclonal antibodies via linkers (1). They are designed to selectively deliver cytotoxic agents to tumor cells where specific tumor-associated antigens are overexpressed on the cell surface, thereby minimizing systemic toxicity. Ado-trastuzumab emtansine (also known as T-DM1) is an antibody–drug conjugate (ADC) approved by the U.S. Food and Drug Administration for the treatment of human epidermal growth factor receptor 2 (HER2)–positive metastatic breast cancer in patients previously treated with trastuzumab and taxane (2). T-DM1 consists of trastuzumab, a humanized monoclonal antibody directed against HER2 and a microtubule inhibitor DM1 that is conjugated to trastuzumab via a thioether linker (3). DM1 is a potent microtubule polymerization inhibitor that induces mitotic arrest and kills tumor cells at sub-nanomolar concentration (4). It is 25- to 270-fold more potent than paclitaxel and 180- to 4,000-fold more potent than doxorubicin (5, 6). However, its side effects and lack of specificity prevented it from clinical use (7).

HER2 is a member of EGFR/ErbB family of receptor tyrosine kinases with significant roles in breast cancer tumorigenesis and is overexpressed in 15-30% of breast cancers (8). Upon binding to HER2, T-DM1 is internalized and processed in lysosomes to release the active catabolite lysine-N(ε)-N-maleimidomethyl-cyclohexane-1-carboxylate (MCC)-DM1 (Lys-MCC-DM1) (3, 9-12). The released Lys-MCC-DM1 exerts anti-microtubule functions via microtubule destabilization which results in mitotic arrest, cell growth inhibition, and cell death (9-12).
T-DM1 significantly prolongs progression-free and overall survival with less toxicity than lapatinib plus capecitabine in patients with HER2-positive advanced breast cancer (13, 14). However, T-DM1 therapy is associated with serious grade 3 or greater adverse events, including hepatotoxicity (13-15). It was reported that all-grade increases in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) occurred in 208 (23.5%) and 139 (15.7%) patients, respectively out of 884 T-DM1-exposed patients. 36 patients (4.1%) had grade 3 increase in serum ALT (4.1%), 2 patients (0.2%) grade 4 increase in serum ALT, and 27 patients (3.1%) grade 3 increase in serum AST (15). Three patients were diagnosed with nodular regenerative hyperplasia (NRH), which resulted in one death due to liver failure. Hepatotoxicity is one of black box warnings on the labeling for the prescription of T-DM1 (http://www.gene.com/download/pdf/kadcyla_prescribing.pdf). However, mechanisms underlying T-DM1-induced hepatotoxicity remain elusive (15). It has not been reported whether T-DM1 directly targets hepatocytes via HER2 to induce hepatotoxicity.

Gemtuzumab ozogamicine (GO) is an ADC consisting of an anti-CD33 monoclonal antibody conjugated to calicheamicin (16). GO was approved in 2000 for the treatment of acute myeloid leukemia (AML) and was withdrawn from the market in 2010 due to product safety and efficacy concerns (17, 18). Among the safety concerns were clinical symptoms of hepatotoxicity, including hepatic veno-occlusive disease, which is a significant GO related toxicity (19). In addition, 29% of patients had Grade 3 or Grade 4 hyperbilirubinemia and 9% had Grade 3 or Grade 4 ALT level abnormalities (20). However, the cause of GO-induced hepatotoxicity remains unproven. Study by Maniecki et al. demonstrated that CD33 receptor, thought to be a specific marker for the cells of the myeloid lineage, is widely distributed in the liver tissue and highly
expressed on hepatocytes (21). This study suggested that the specific targeting of hepatocytes by GO resulted in the accumulation of antibody-toxin conjugates in hepatocytes causing calicheamicin-induced damage (20). Besides GO, signs of liver dysfunction were also identified in other ADC therapies in clinical testing (22-24). Given the significant clinical potential of ADC therapies and the substantial increase in number of ADCs in clinical trials, it is critical to obtain a better understanding of mechanisms by which ADCs, including T-DM1, induce hepatotoxicity.

Murine model has been widely used to study the mechanisms of trastuzumab-induced cardiotoxicity (25-29). Riccio et al have shown that trastuzumab binds to mouse HER2 (26) and that mice treated with trastuzumab have reduced left ventricular ejection fraction (LVEF) (25-29). Trastuzumab may directly block anti-apoptotic signaling, leading to premature cardiac dysfunction (28). Although HER2 is present at a low level in mouse cardiomyocytes, the studies from different laboratories suggested that trastuzumab-induced cardiotoxicity may be HER2-dependent (25-29). Furthermore, trastuzumab treatment induces apoptosis in cardiac sections of heart tissues from mice treated with trastuzumab (25, 28). In this study, we used the cellular and murine models to investigate the mechanisms by which T-DM1 induces hepatotoxicity. We aimed to evaluate HER2 expression in mouse and human hepatocytes and T-DM1-induced endocytosis. We also examined its associated intracellular damage, and liver injury in mice treated with T-DM1.

**Materials and Methods**

**Cell lines and control DM1 ADC**
Human hepatocytes (THLE2) and mouse hepatocytes (AML12) were obtained from ATCC and used within six months after cell lines were ordered and cultured in BEGM SingleQuots medium containing 10% fetal bovine serum (FBS) and DMEM/F-12 (1:1) mixture containing 10% FBS, respectively. Human primary hepatocytes (HPH) were obtained from ScienCell and used within the six months after the cells were ordered. THLE2, AML12 and HPH were not authenticated in our laboratory. αHFc-NC-DM1 is anti-human IgG Fc specific antibody conjugated to maytansinoid DM1 with a non-cleavable linker. αMFc-NC-DM1 is anti-mouse IgG Fc specific antibody conjugated to maytansinoid DM1 with a non-cleavable linker. Both HFc-NC-DM1 and MFc-NC-DM1 were purchased from Moradec LLC.

**Immunofluorescence microscopy**

For LAMP1 and microtubule-staining, cells were plated on fibronectin (10 µg/ml, Sigma-Aldrich) pre-coated glass coverslips (12 mm; Fisher Scientific) in 12-well plate (Corning) and cultured overnight. After washing with pre-warmed DPBS twice, cells were fixed in 4% paraformaldehyde (PFA, Electron Microscopy Sciences) in DPBS for 15 min and permeabilized with 0.2% Triton X-100 in DPBS for 10 min. The samples were washed with DPBS and blocked with 2% bovine serum albumin (BSA) in DPBS at room temperature for 1-2 hours. Anti-α-tubulin (clone DM1A, Sigma-Aldrich) or anti-LAMP1 (BD Biosciences) antibodies were diluted 1:100 in the blocking solution, and the samples were incubated with these antibodies at 4°C overnight. After washing with DPBS three times, the samples were incubated with Alexa Fluor 488 or 594-conjugated anti-mouse secondary antibodies (1:100 dilution, Life Technologies) in the blocking solution at the room temperature for 1 hour. After washing with DPBS for 10 min three times, the coverslips were mounted inverted on glass slides with Prolong gold antifade reagent containing...
DAPI (Life Technologies, Grand Island, NY). Stained samples were imaged on confocal LSM 510 Meta microscope (Carl Zeiss Microscopy). For immunostaining using trastuzumab of T-DM1 as the primary antibody, cells on fibronectin pre-coated cover glass were fixed in 4% PFA in cell culture media and then were permeabilized with 0.5% saponin (EMD Millipore, MA) in TBS at room temperature for 15 min, and washed with TBS for 5 min three times. The samples were blocked with 10% goat serum (Jackson ImmunoResearch) in TBS at 37°C for 30 min and then incubated with the purified human IgG (Life Technologies), trastuzumab or T-DM1 antibodies (each at 50 μg/mL) at 37°C for 1h. After washing with TBS containing 1% goat serum three times (10 min for each wash), the samples were incubated with Alexa Fluor 488-conjugated anti-human secondary antibody (1:100 dilution, Life Technologies) diluted in TBS at 37°C for 30 min. The samples were washed with TBS containing 1% goat serum at room temperature three times and then mounted on glass slides as described above. Images were captured on an LSM 510 Meta confocal microscope attached to an Axiovert 200 inverted microscope (Carl Zeiss).

**Immunohistochemistry (IHC)**

Livers from healthy C57BL/6 mice were harvested, fixed and embedded in paraffin. IHC staining was performed by Histoserv, Inc. According their IHC protocol, the tissue sections were treated at 90°C for 20 min for antigen retrieval and then were treated with hydrogen peroxide and blocked with BSA. After washing with TBST, the sections were incubated with control rabbit IgG (Life Technologies) or rabbit anti-HER2 antibody (29D8, 1:400 dilution, Cell Signaling Technology) at room temperature overnight. After TBST washing, the sections were incubated with secondary antibody for 30 min at room temperature and then streptavidin-HRP incubation for 30 min at room temperature. After washing with TBST the slides were developed with DAB.
(Diaminobenzidine Tetrahydrochloride). Images of the IHC staining were collected using Pannoramic MIDI Digital Slide Scanner (3DHISTECH Ltd.).

**Duolink proximity ligation assay**

Duolink proximity ligation assay (PLA) was performed according to the instructions obtained from the manufacturer’s website ([http://www.olink.com/products/duolink/downloads/duolink-manuals-and-guidelines](http://www.olink.com/products/duolink/downloads/duolink-manuals-and-guidelines)). Briefly, the cells were fixed and permeabilized and incubated with primary antibodies from two different species recognizing either C-terminal region of HER2 (rabbit polyclonal) or DM1 component of T-DM1 (mouse monoclonal). As a control, cells that were not treated with T-DM1, but stained for HER2 and DM1, were used. Controls also included no primary antibodies. After washing and permeabilization step, the cells were incubated with secondary antibodies with the ligated oligonucleotide probes (PLA plus and PLA minus) for 1 hour at 37°C, followed by ligation and amplification reactions. In the amplification step, fluorescently labelled oligonucleotides were added together with polymerase. The signals from fluorescently labelled amplified concatemeric product were visualized by fluorescent microscopy. Distinct single fluorescent spots indicate interaction between HER2 protein and T-DM1.

**Animal model**

All animal experiments were approved by and conducted in accordance with the regulations of the FDA IACUC guidelines. The first cohort of thirty six C57BL/6 mice age 8-9 weeks (National Cancer Institute, Frederick MD) was randomly assigned into three groups to receive a single tail vein injection of vehicle (6% sucrose, 0.02% Tween 20 and 10 mM Sodium succinate), trastuzumab (Genentech, Inc), or T-DM1 (Genentech, Inc). Both trastuzumab and T-DM1 were
purchased from the pharmacy at the National Institutes of Health (NIH), Bethesda, MD. Twelve mice in the T-DM1 group received 30 mg/kg of T-DM1, twelve mice in trastuzumab group received 29.4 mg/kg trastuzumab (comparable mole quantity of trastuzumab to T-DM1), and twelve mice in the sham group received a comparable volume of vehicle. Mice were bled 10 days prior to the drug injection to establish a base-line for serum biomarkers. Mice were then bled and sacrificed on days 1, 3 or 7 after the tail vein injection of vehicle, trastuzumab and T-DM1. Livers from each mouse were harvested for H&E staining and electron microscopy (EM) study.

The second cohort of 40 C57BL/6 mice was randomly assigned into five groups, including vehicle, trastuzumab (29.4 mg/kg), T-DM1 (3 mg/kg), T-DM1 (10 mg/kg) or T-DM1 (30 mg/kg), for the time and dose dependent study. There were eight mice in each group. Mice received a single tail vein injection of vehicle, trastuzumab or T-DM1. Mice were bled and then euthanized 12h or 72h post tail vein injection. Livers from mice were harvested for H&E staining, TNFα gene expression and EM study. Livers from healthy C57BL/6 mice were harvested for immunohistochemistry (IHC) and Western blot analysis.

**Serum hepatic markers**

Blood collection and serum isolation: Tail vein nick was performed during survival blood collection, and terminal cardiac blood collection was used during euthanasia. ALT, AST and LDH serum test was conducted in the chemistry laboratory at NIH clinical center using the same produces as those used for human clinical samples.

**Reverse transcription and quantitative PCR**
Total RNA was isolated from freshly frozen liver tissues and reverse transcriptase Superscript II (Life Technologies) was used for cDNA synthesis according to the manufacturer’s protocol. Quantitative PCR was performed using Taqman Gene Expression PCR Master Mix and commercially available primers for genes with the 7900 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA). Actin was used as reference to normalize the expression level.

Electron Microscope

Fresh liver samples were fixed for 1 hour at room temperature and then overnight at 4°C in TEM/SEM tousimis® Glutaraldehyde, 2.5% in 0.1M Na-Cacodylate Buffer (Tousimis, Rockville MD), osmicated for 1 hour at the room temperature in the same buffer, en bloc stained with 0.5% uranyl acetate for 1 hour, dehydrated in a graded ethanol series, embedded in Epon 812 substitute, and examined on a Hitachi H7650 transmission electron microscope.

Mitochondria membrane potential assay

Cells were plated on fibronectin pre-coated glass coverslips in 12-well plate and cultured overnight. Next day cells were treated with T-DM1 (0 or 20 µg/ml) and then incubated for 2 days. Mitochondrial Transmembrane Potential Apoptosis Detection Kit (Abcam) was used according to the manufacturer’s protocol. Images were captured by EVOS FL system (Life Technologies). According to the manufacturer’s instructions, the kit utilizes a cationic dye that fluoresces differently in healthy vs. apoptotic cells. In healthy cells, the dye accumulates and aggregates in the mitochondria with bright fluorescence, whereas in apoptotic cells, the dye cannot aggregate in mitochondria due to the altered mitochondrial transmembrane potential.

Alexa Fluor 488-conjugation
Alexa Fluor 488 Protein Labeling Kit (Life Technologies) was used for the labeling of T-DM1 with Alexa Fluor 488. Briefly, 2.5 mg T-DM1 (5 mg/ml, 0.5 ml in DPBS) was conjugated with Alexa Fluor 488 according to the manufacturer’s instructions. The kit has a tetrafluorophenyl (TFP) ester moiety, and it reacts efficiently with primary amines of T-DM1 to form stable dye-protein conjugate. The Alexa Fluor 488-labeled T-DM1 was eluted by serum-free BEGM medium.

**Western Blotting and Immunoprecipitation**

Whole cell lysates (WCL) of THLE2 or AML12 cells were used either for detection of HER2 protein expression or immunoprecipitation experiments. For immunoprecipitation, WCL were incubated with control IgG (human IgG, Life Technologies), trastuzumab, T-DM1 or anti-HER2 antibody (29D8, Cell Signaling Technology). The immuneprecipitated HER2 was detected using anti-HER2 antibody (29D8), which recognizes both mouse and human HER2.

**Statistical analysis**

GraphPad Prism was used for statistical studies. Statistical significance was determined by Student’s t-test (*, P < 0.05; **, P < 0.01). Data are expressed as mean ± SEM.

**Results**

**HER2 is expressed in mouse and human hepatocytes, and T-DM1 associates with HER2 on the hepatocyte cell surface**

To investigate the possibility that specific targeting of hepatocytes via HER2 may contribute to hepatotoxicity induced by T-DM1, HER2 expression in human and mouse
hepatocytes and mouse liver tissue was analyzed by Western blot. Figure 1A showed HER2 expression in human hepatocyte (THLE2) vs. human primary hepatocytes (HPH) (Left panels) and in THLE2 vs. mouse hepatocyte (AML12) (Right panels). HER2 was detected in liver tissues from three mice by Western blot analysis (Figure 1B). HER2 expression in HER2-positive breast cancer cells (SKBR3) and AML12 was used as controls (Figure 1B). Immuno-precipitation study (upper panel) showed that both trastuzumab and T-DM1 bound to human HER2 (Figure 1C) consistent with previous report (30). Figure 1C (lower panel) showed that both trastuzumab and T-DM1 were capable of immunoprecipitating HER2 from WCL of mouse hepatocytes although the binding affinity of trastuzumab and T-DM1 to mouse HER2 was lower than that of mouse anti-HER2 antibody (29D8). Using the immunofluorescence approach, we found that HER2 was expressed in both plasma membrane and inside of cells in human primary hepatocytes (Figure 1D). A positive HER2 staining by immunohistochemistry (IHC) was observed in mouse liver tissue. HER2 expression by IHC in human liver tissues was reported previously (The Human Protein Atlas: www.proteinatlas.org).

We next examined the interaction of trastuzumab or T-DM1 with HER2 in hepatocytes. As shown in Figure 1F (arrowheads), the strong positive signal was detected at the cell edges of both human and mouse hepatocytes when either trastuzumab or T-DM1 was used for detection of cell surface HER2, suggesting that both trastuzumab and T-DM1 bind to HER2 expressed on the cell surface. In contrast, control IgG did not give rise to any positive signal on the cell surface (Figure 1F). Using Duolink proximity ligation assay (PLA), we further confirmed that T-DM1 bound to HER2 expressed in hepatocytes (Figure 1G).
T-DM1 is internalized into the human hepatocytes, resulting in disorganized microtubule networks, nucleus fragmentation/multiple nuclei and cell growth inhibition

We next addressed the question of whether T-DM1 was capable of inducing endocytosis upon binding to HER2 expressed on hepatocyte cell surface. Human hepatocytes (THLE2) were incubated with Alexa Fluor 488-conjugated T-DM1 for 1 hr. The co-localization of the Alexa Fluor 488-conjugated T-DM1 with the lysosomal marker LAMP1 was examined. As shown in Figure 2A, Alexa Fluor 488-conjugated T-DM1 was internalized into THLE2 cells and co-localized with LAMP1 (arrows), indicating that T-DM1 has the ability to induce endocytosis when it binds to HER2.

After internalization of the receptor-T-DM1 complex, intracellular release of DM1-containing moieties from T-DM1 occurs following lysosomal degradation of trastuzumab component. We next tested whether T-DM1 induces microtubule destabilization and the mitotic arrest leading to growth inhibition. As shown in Figure 2B, after cells were incubated with T-DM1 for 48h, microtubule structures in THLE2 cells were disorganized (white arrow) as compared with that in the untreated cells (orange arrow). Furthermore, multiple nuclei and fragmented nucleus were also observed in the cells treated with T-DM1 (Figure 2B, red arrows), indicating that the internalized T-DM1 prevented mitosis of THLE2 cells and induced apoptosis. Figure 2B (graph) showed growth profiles of THLE2 cells treated with T-DM1 or left untreated and demonstrated that the growth of hepatocyte was inhibited by T-DM1 in a dose dependent manner. It should be noted that the inhibition of cell growth by T-DM1 shown in Figure 2B was not due to possible residual free DM1 that may be present in the T-DM1 solution since after immuno-depletion of T-DM1 from the solution using Protein A/G agarose, the supernatant did not exhibit any inhibitory effect on cell growth (data not shown). Figure 2C also demonstrated that T-DM1
was capable of destabilizing microtubule structures (white arrow), inducing multi-nuclei in mouse hepatocytes (red arrows), and inhibiting AML12 cell growth (Figure 2C, graph). Furthermore, as shown in Figure 2D and E, the ability to inhibit THLE2 cell growth by non-HER2 targeting control ADCs ($\alpha$HFc-NC-DM1 and $\alpha$MFc-NC-DM1) was significantly reduced as compared to T-DM1, indicating that the T-DM1-induced cytotoxicity is mainly HER2-dependent in this in vitro toxicology study. Taken together, these data suggest that T-DM1 can specifically target both human and mouse hepatocytes via HER2, resulting in DM1-associated intracellular damages.

**T-DM1 treatment increases the serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH), and TNF$\alpha$ gene expression in liver tissue.**

Data presented in Figures 1, and 2 suggest that mouse can be used as an animal model to study the mechanisms of T-DM1-induced hepatotoxicity. We next investigated whether T-DM1 treatment induced acute liver injury in mice. T-DM1 was administered in a single tail vein injection, and time-dependent effects of T-DM1 on liver function were evaluated by measuring AST, ALT, and lactate dehydrogenase (LDH) in the serum. Consistent with the previous report (30), a single dose of T-DM1 at 30 mg/kg was tolerated in mice (data not shown). While mice administered control vehicle or trastuzumab at 29.4 mg/kg showed no significant increases in ALT, AST and LDH at three different time points (Day 1, 3, and 7), mice administered T-DM1 showed significant increases in serum levels of ALT, AST, and LDH compared with that of control or trastuzumab-treated mice (Figure 3 A, B, and C). It has been shown that tissue macrophages such as Kupffer cells respond to the liver injury and secret pre-inflammatory cytokines such as
tumor necrosis factor alpha (TNFα) (31-33). The cytokines can further promote the accumulation of neutrophils in liver leading to serious hepatic damage (32, 33). Thus, we measured TNFα gene expression in liver tissues from mice treated with control vehicle, trastuzumab or T-DM1 at different time points, and found that the TNFα gene expression was significantly elevated in mice treated with T-DM1 as compared with those treated with control vehicle or trastuzumab (Figure 3D). We observed a downward trend in the serum levels of TNFα from Day 1 to Day 7 (Figure 3D).

We next addressed whether T-DM1 induced the elevation of serum ALT, AST and LDH in a dose dependent manner. As shown in Figure 3E, the significant increases in serum levels of ALT and AST, but not LDH, were observed in mice treated with the highest dose of T-DM1 (30 mg/kg) at the 12h post T-DM1 injection. At 72h post-treatment of T-DM1, the serum levels of ALT, AST and LDH were significantly increased at the dose of 30 mg/kg of T-DM1 (Figure 3F), consistent with data shown in Figure 3B. The serum levels of ALT and LDH, but not AST, were also significantly increased at the dose of 10 mg/kg of T-DM1, and there were no significant increases in ALT, AST and LDH at the dose of 3 mg/kg of T-DM1 (Figure 3F). These data indicated that T-DM1-induced liver injure was in a dose dependent manner. Surprisingly, the serum levels of ALT were also increased in mice treated with trastuzumab at 72h post-injection as compared with control mice (Figure 3F).

**T-DM1 treatment induces inflammation and necrosis in liver tissues**

The hallmarks of acute hepatocellular injury are inflammation and/or necrosis (34). Both inflammation (green arrows) and necrosis (yellow arrows) were found in liver tissues from the
mice treated with T-DM1 at the dose of 30 mg/kg, whereas inflammation and necrosis were not observed in liver tissues from control mice (Table 1A, Figure 4A, C and D). Inflammation, but not necrosis, was noticed in liver tissue in one mouse treated with trastuzumab at the dose of 29.4 mg/kg on day 7 (Table 1A, Figure 4B, green arrow). However, the reason that caused inflammation remains unclear. T-DM1 induced necrosis in liver tissues was observed as early as 12h post-T-DM1 treatment at the doses of 10 mg/kg and 30 mg/kg, but not at 3 mg/kg. 72h post-T-DM1 treatment, necrosis was found in all tissue samples obtained from the mice treated with three different doses of T-DM1 (Table 1B). No necrosis was found in liver tissues obtained from the mice treated with vehicle control or trastuzumab (Table 1B).

**T-DM1 induces the outer mitochondrial membrane rupture and mitochondrial membrane potential dysfunction**

The outer mitochondrial membrane rupture (OMR), which releases mitochondrial proteins of the intermembrane space into the cytoplasm, has been implicated as the central event of the mitochondrial-dependent apoptosis (35). These mitochondrial proteins initiate and execute the apoptotic processes (35). Electron microscopy (EM) study demonstrated that outer mitochondrial membrane rupture was frequently observed in T-DM1-treated liver cells, while control and trastuzumab-treated liver cells showed no damages in cell organelle structures (Figure 5A, red arrows). To confirm the observation from the mice, we also examined whether mitochondrial dysfunction could be found in hepatocytes treated with T-DM1. As shown in Figure 5B, using Mitochondrial Transmembrane Potential Apoptosis Detection Kit, the cationic dye (fluorescence dots) accumulated in the cells that were not treated with T-DM1, whereas the dye accumulation in mitochondria was inhibited in cells treated with T-DM1, suggesting
that T-DM1 induced mitochondrial membrane dysfunction in hepatocytes. Taken together, these data indicate that T-DM1 induced apoptosis of hepatocytes \textit{in vivo} and \textit{in vitro}.

\section*{Discussion}

Hepatotoxicity is widely regarded as the leading cause of failure of drug development programs and a serious safety concern in clinical studies and post-marketing surveillance (36, 37). In the analysis of drugs withdrawn for toxicity reasons in the period between 1992 and 2002, hepatotoxicity was identified in 27\% of cases (36). While mechanisms underlying most instances of drug-induced hepatotoxicity are still not well understood, different studies implicated factors such as host metabolism, detoxification, liver-regeneration and immune response pathways (38, 39).

Hepatotoxicity has been reported in several other ADC therapies currently undergoing different stages of clinical testing (22-24). In a phase 1 study of cantuzumab mertansine, hepatotoxicity (reversible elevations of hepatic transaminases and occasionally alkaline phosphatase and bilirubin) were identified as principal toxic side effects of the therapy (22). In a phase I study of inotuzumab ozogamicine, increased aspartate aminotransferases and bilirubinemia were reported for 18.4\% and 22.4\% of study participants, respectively (23). In a phase 1 study of MLN2704, which is an ADC designed to deliver DM1 to prostate-specific membrane antigen (PSMA)-expressing cells, in patients with progressive metastatic castration-resistant prostate cancer, abnormal hepatic functions were identified in more than 20\%
study participants (24). Given the significant clinical promise of ADC therapies, better understanding of the molecular basis for hepatotoxicity induced by ADC, including T-DM1, will benefit ADC clinical development programs, as well as post-marketing surveillance.

Thrombocytopenia is the dose-limiting toxicity induced by T-DM1 (13, 40). A study conducted by Uppal et al. suggests that human megakaryocytes (MKs) internalize T-DM1 in a HER2-independent, FcγRIIa-dependent manner, resulting in intracellular release of DM1 (40). However, the mechanism of DM1 release remains elusive since the authors were unable to detect co-localization between the internalized T-DM1 and lysosomal LAMP1 by immunofluorescence. Thon and colleagues reported that T-DM1-induced thrombocytopenia occurred via a mechanism that is both HER2 and FcγRIIa independent due to the fact that mouse megakaryocytes and platelets do not express HER2 and FcγRIIa and that both take up T-DM1 (41). This study suggests that T-DM1 is endocytosed through an alternative pathway (41). While it is still a matter of debate whether trastuzumab associates with mouse HER2, many laboratories have been using mouse model to investigate the mechanisms of trastuzumab-induced cardiotoxicity, and data from these studies suggest that trastuzumab-induced cardiotoxicity is HER2 dependent (25-29). We used both cellular and mouse model systems to investigate a clinically relevant case of drug-induced hepatotoxicity and to elucidate the possible mechanisms of T-DM1-induced hepatotoxicity. We found that HER2 was expressed in mouse and human hepatocytes, as well as mouse liver and that T-DM1 was able to associate with mouse and human HER2, although binding affinity of trastuzumab or T-DM1 to mouse HER2 is lower than that of human HER2. We also demonstrated that T-DM1 was capable of mediating endocytosis and that the internalized T-DM1 co-localized with lysosomal LAMP1, induced destabilization of
microtubules and inhibited hepatocyte growth, indicating that DM1 was released in lysosomes and that the released T-DM1 induced cytotoxicity in hepatocytes. According to the public resource of data (www.proteinatlas.org), FcγRIIa, FcγRIIb, FcγRIIIa are not expressed in hepatocytes (42), suggesting that T-DM1 internalization is unlikely mediated by Fc receptors. Based on data we have, we propose a novel mechanism by which T-DM1 directly targets hepatocytes via HER2 contributing to T-DM1-induced hepatotoxicity.

Results from our cellular model provide scientific rationale to use mouse as a relevant animal model to further investigate mechanisms of T-DM1-induced hepatotoxicity. Our animal data reveal some important findings. First of all, T-DM1-induced hepatotoxicity is dose dependent. Secondly, trastuzumab was found to induce inflammation, but not necrosis, in liver tissue. Trastuzumab-induced inflammation was recovered within three days, except one mouse in the first cohort (Table 1B). Thirdly, T-DM1-induced hepatocellular injury contains both inflammation and necrosis (H&E staining) with significant liver function damage, including increase in serum levels of ALT, AST, and LDH. Fourthly, TNFα gene expression in liver tissue is significantly increased in mice treated with T-DM1 as compared with that treated with trastuzumab or vehicle. TNFα is a pro-inflammatory cytokine capable of inducing apoptosis. Cytokine-mediated pro-apoptotic signaling is an important component in the pathophysiology of drug induced liver injury (43, 44). It has been reported that TNFα severely enhances liver damage caused by various xenobiotics (43, 45-47) and is the major cytokine to be excreted by the liver stationary macrophages (Kupffer cells) in response to hepatocyte damage (33). It is possible that T-DM1-mediated secretion of TNFα, which activates pro-apoptotic signaling pathway, significantly enhances the liver damage that is initially caused by DM1-mediated
intracellular stress. The mechanisms by which T-DM1 induces TNFα-mediated apoptosis are further supported by our EM study in that T-DM1 induces the outer mitochondrial membrane rupture, an indication of mitochondrial-dependent apoptosis. Our work sheds new light on the mechanism by which T-DM1 induces hepatotoxicity, which may yield novel strategies to manage T-DM1, as well as other ADCs-induced liver toxicity.

Disclaimer

This article reflects the views of the author and should not be construed to represent FDA’s views or policies.

Acknowledgements

We thank Drs. Tao Wang and Eric Hales of FDA for critical internal review of this manuscript.

We thank Dr. George Kunos, the scientific director of NIAAA, NIH, for his support.

References


Table 1. T-DM1 causes hepatocellular injury in mouse liver tissues

A.

<table>
<thead>
<tr>
<th></th>
<th>Hepatocellular injury</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td>Control$^a$</td>
<td>No histopathological changes</td>
</tr>
<tr>
<td>Trastuzumab$^b$</td>
<td>No histopathological changes</td>
</tr>
<tr>
<td>T-DM1$^c$</td>
<td>2 mice with liver necrosis</td>
</tr>
</tbody>
</table>

$^a$ Liver tissue from 1 mouse was randomly tested for H&E staining at each time point.

$^b$ Liver tissues from 2 mice were randomly tested for H&E staining at each time point.

$^c$ Liver tissues from 2 mice were randomly tested for H&E staining at each time point.
Table 1 Hepatocellular injury in liver tissues was evaluated by frequency of inflammation and necrosis induced by the vehicle, trastuzumab, and T-DM1. (A). The first cohort of thirty six C57/BL6 mice was randomly assigned into three groups to receive a single tail vein injection of vehicle (6% sucrose, 0.02% Tween 20 and 10 mM Sodium succinate), trastuzumab (29.4 mg/kg),

<table>
<thead>
<tr>
<th>Hepatocellular injury</th>
<th>12 hours</th>
<th>72 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1 of 4 mice with liver inflammation</td>
<td>1 of 4 mice with liver inflammation</td>
</tr>
<tr>
<td>Trastuzumab</td>
<td>4 mice with liver inflammation</td>
<td>No histopathological changes</td>
</tr>
<tr>
<td>T-DM1 3 mg/kg</td>
<td>3 of 4 mice with liver inflammation</td>
<td>4 mice with liver necrosis</td>
</tr>
<tr>
<td>T-DM1 10 mg/kg</td>
<td>4 mice with liver necrosis</td>
<td>4 mice with liver necrosis</td>
</tr>
<tr>
<td>T-DM1 30 mg/kg</td>
<td>2 of 4 mice with liver necrosis</td>
<td>4 mice with liver necrosis</td>
</tr>
</tbody>
</table>

All groups contained 4 mice tested for H&E staining at each time point, except trastuzumab 72h group in that 3 mice were tested for H&E staining.
or T-DM1 (30 mg/kg). At the indicated times, mice were sacrificed and livers were harvested for H&E staining. (B). The second cohort of 40 C57/BL6 mice was randomly assigned into five groups to receive a single tail vein injection of vehicle, trastuzumab (29.4 mg/kg), T-DM1 (3 mg/kg), T-DM1 (10 mg/kg) or T-DM1 (30 mg/kg). At the indicated times, mice were sacrificed, and livers were harvested for H&E staining.

**Figure legends:**

Figure 1. HER2 is expressed in human and mouse hepatocytes and mouse liver tissues, and both trastuzumab and T-DM1 bind to HER2 on the cell surface of hepatocytes

(A) The levels of endogenous HER2 in whole cell lysates (WCL) of human and mouse hepatocytes (THLE2 and AML12, respectively) and human primary hepatocytes (HPH) were determined by Western blot analysis using anti-HER2 antibody (29D8). (B) The levels of endogenous HER2 in WCL of mouse hepatocytes (AML12) (20 µg) and mouse liver tissue (20 µg) were determined by Western blot analysis using anti-HER2 antibody (29D8). (C) Endogenous HER2 in WCL collected from THLE2 and AML12 was immunoprecipitated using human control IgG, trastuzumab, T-DM1 and anti-HER2 antibody (29D8). Immunoprecipitated HER2 protein was detected using anti-HER2 antibody (29D8). (D) Endogenous HER2 in human primary hepatocytes was detected by anti-HER2 antibody (29D8) using immune-fluorescence approach. Bar, 50 µm. (E) Endogenous HER2 in mouse liver tissues was detected by Immunohistochemistry (IHC) staining. Bar, 50 µm. (F) Binding of trastuzumab and T-DM1 to the cell surface HER2 of THLE2 and AML12 was detected using immunofluorescence approach. Human IgG was used as a negative control. Bar, 20 µm. (G) THLE2 cells were plated overnight, and then either control treated with IgG (50 or 200 µg/ml) or treated...
with T-DM1 (50 or 200 µg/ml) for 1 hour. Following treatment, the cells were fixed and subjected to Duolink proximity ligation assay (PLA) according to manufacturer’s instructions (see materials and methods section). Upper images: cells which were not treated with T-DM1, but stained with both primary and both secondary antibodies (Right). Middle images: T-DM1 treated cells which were not stained with either primary antibodies, but stained with both secondary antibodies. Bottom images: T-DM1 treated cells which were stained with the both primary and both secondary antibodies. Bar, 50 µm.

**Figure 2. T-DM1 is internalized leading to the disorganized microtubules, multiple and fragmented nuclei, and cell growth inhibition in THLE2 and AML12 cells**

(A) Human hepatocytes (THLE2) were seeded on fibronectin pre-coated glass coverslips overnight and then were incubated with Alexa Fluor 488-conjugated T-DM1 for 1 hr. Cells were then fixed, permeabilized, and stained for LAMP1 (red). Merge (red arrows), Alexa Fluor 488-conjugated T-DM1 (green) and lysosomal marker LAMP1 colocalized. Nuclei were stained with DAPI (blue). Bar, 20 µm. (B) THLE2 cells were plated on fibronectin pre-coated glass coverslips and treated with T-DM1 overnight or left untreated. Cells were then fixed, permeabilized and stained for microtubules (green). Nuclei were stained with DAPI (blue). White arrows, disorganized microtubules. Red arrows, nucleus fragmentation/multiple nuclei. Graph, growth profiles of cells treated with indicated concentrations of T-DM1. Cells were seeded at 0.5 x 10^5, harvested at the indicated times and counted. Data are the mean ± SEM of three independent experiments in triplicate. Bar, 50 µm. (C) The experimental procedures were essentially the same as described in Figure 2B except AML12 cells were seeded at 0.25 x 10^5. Bar, 50 µm. (D, E) Graph, growth profiles of THLE2 cells treated with either 5 µg/ml (D) or 10 µg/ml (E) of T-DM1, αHFc-
NC-DM1 or αMFC-NC-DM1. The THLE2 cells were seeded at 0.5 x 10^5, harvested at the indicated times and counted. Data are the mean ± SEM (** p < 0.01).

**Figure 3. T-DM1 treatment increases the serum levels of AST, ALT and LDH, and the gene expression of TNFα in liver tissues**

(A-C) C57BL/6 mice were administered a single tail vein injection of vehicle, trastuzumab (29.4 mg/kg) or T-DM1 (30 mg/kg), and then bled and sacrificed on day 1, 3 or 7. The serum levels of ALT, AST, and LDH were determined. Data are the means ± SEM of 4 mice (* p < 0.05).

(D) TNFα gene expression in the freshly frozen liver tissues was determined using Taqman Gene Expression PCR Master Mix. Actin was used as reference to normalize the expression level. Data are the means ± SEM of 4 mice (* p < 0.05).

(E-F) C57/BL6 mice were administered a single tail vein injection of vehicle, trastuzumab (29.4 mg/kg) or T-DM1 (3, 10 or 30 mg/kg). At the indicated times, mice were bled and then euthanized. The serum levels of ALT, AST, and LDH were determined. Data are the means ± SEM of 4 mice (* p < 0.05).

**Figure 4. T-DM1 treatment induces inflammation and necrosis in mouse liver**

Liver tissues obtained from the mice (first cohort) treated with vehicle control (A), trastuzumab (B) and T-DM1 (C and D) were collected for H&E staining. Green arrows: inflammation; yellow arrows: necrosis. Bar, 50 µm.

**Figure 5. T-DM1 induces the outer mitochondrial membrane rupture and mitochondrial membrane potential dysfunction**
(A) Electron microscopy images of liver tissues from mice treated with control, trastuzumab (29.4 mg/kg) or T-DM1 (30 mg/kg). Red arrows, outer mitochondrial membrane rupture. Bar, 1 μm.

(B) Images of a cationic dye accumulated in cells (punctated fluorescence dots). Left, image from the cells (THLE2) left untreated; Right, image from the cells treated with T-DM1 (20 μg/mL). Bar, 50 μm.
Figure 2

A) LAMP1, T-DM1, Nucleus, Merge

B) THLE2

C) AML12

D) No treatment, T-DM1, αHFc-NC-DM1, αMFc-NC-DM1

E) No treatment, T-DM1, αHFc-NC-DM1, αMFc-NC-DM1

** (5 µg ADC/ml) (10 µg ADC/ml) on April 13, 2017. © 2015 American Association for Cancer Research. mct.aacrjournals.org Downloaded from mct.aacrjournals.org on April 13, 2017. © 2015 American Association for Cancer Research.
Figure 3

A. 1 day

B. 3 days

C. 7 days

D. TNFα

E. 12h

F. 72h

Legend:
- control
- Trastuzumab 29.4 mg/kg
- T-DM1 30 mg/kg
- T-DM1 3 mg/kg
- T-DM1 10 mg/kg
- T-DM1 30 mg/kg

Alt, AST, LDH levels over time with different treatments.
Figure 4

A  Control

B  Trastuzumab

C  T-DM1

D  T-DM1
Molecular Cancer Therapeutics

Ado-trastuzumab emtansine targets hepatocytes via human epidermal growth factor receptor 2 to induce hepatotoxicity

Haoheng Yan, Yukinori Endo, Yi Shen, et al.

Mol Cancer Ther Published OnlineFirst December 28, 2015.

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Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-15-0580

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