Liver Microvascular Injury and Thrombocytopenia of Antibody-Calicheamicin Conjugates in Cynomolgus Monkeys – Mechanism and Monitoring

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Liver and Platelet Effects of Calicheamicin Conjugates


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Translational relevance

Gemtuzumab ozogamicin (Mylotarg®) and inotuzumab ozogamicin are antibody-calicheamicin conjugates developed for treatment of acute myeloid leukemia and acute lymphoblastic leukemia, respectively. Clinical adverse reactions with these drugs include thrombocytopenia and liver toxicity, characterized by increases in serum aminotransferases and bilirubin with occasional cases of hepatic sinusoidal obstruction syndrome. This experimental work in cynomolgus monkeys demonstrates a relationship between platelet and liver effects, through an initial damage to liver sinusoidal endothelial cells that is associated with platelet sequestration in liver sinusoids. This toxicity mechanism may also operate for other non-calicheamicin antibody-drug conjugates (ADCs) with similar adverse events of thrombocytopenia and liver microvascular injury. Evaluation of liver toxicity biomarkers suggested that serum hyaluronic acid may be a sensitive mechanism-based diagnostic marker. This report may have implications for patient care as it provides basis for research into new monitoring strategies, prevention approaches and treatment options through better understanding of ADC toxicities.

Keywords

Antibody-drug conjugate, gemtuzumab ozogamicin, inotuzumab ozogamicin, thrombocytopenia, sinusoidal obstruction syndrome, hyaluronic acid, cynomolgus monkey
Abstract

Purpose: Adverse reactions reported in patients treated with antibody-calicheamicin conjugates such as gemtuzumab ozogamicin (GO, Mylotarg®) and inotuzumab ozogamicin (IO) include thrombocytopenia and sinusoidal obstruction syndrome (SOS). The objective of this experimental work was to investigate the mechanism for thrombocytopenia, characterize the liver injury and identify potential safety biomarkers.

Experimental design: Cynomolgus monkeys were dosed intravenously at 6 mg/m²/dose once every 3 weeks with a non-binding antibody-calicheamicin conjugate (PF-0259) containing the same linker-payload as GO and IO. Monkeys were necropsied 48 hours after the 1st administration (Day 3) or 3 weeks after the 3rd administration (Day 63).

Results: PF-0259 induced acute thrombocytopenia (up to 86% platelet reduction) with nadirs on Days 3-4. There was no indication of effects on megakaryocytes in bone marrow or activation of platelets in peripheral blood. Microscopic evaluation of liver from animals necropsied on Day 3 demonstrated midzonal degeneration and loss of sinusoidal endothelial cells (SECs) associated with marked platelet accumulation in sinusoids. Liver histopathology on Day 63 showed variable endothelial recovery and progression to a combination of sinusoidal capillarization and sinusoidal dilation/hepatocellular atrophy, consistent with early SOS. Among biomarkers evaluated, there were early and sustained increases in serum hyaluronic acid (HA) that correlated well with serum aspartate aminotransferase and liver microscopic changes, suggesting that HA may be a sensitive diagnostic marker of the liver microvascular injury.
**Conclusion:** These data support the conclusion that target-independent damage to liver SECs may be responsible for acute thrombocytopenia (through platelet sequestration in liver sinusoids) and development of SOS.
Introduction

Gemtuzumab ozogamicin (Mylotarg®, GO) and inotuzumab ozogamicin (IO) are antibody-drug conjugates (ADCs) comprised of different humanized monoclonal antibodies and of the same linker and payload (1). The specific payload is a calicheamicin derivative that binds to the DNA minor groove and induces double-stranded DNA breaks. It is conjugated to the antibodies via an acid-labile linker that ensures calicheamicin release at the acidic pH of lysosomes after ADC internalization into target-expressing cells (1). In terms of target specificity, GO binds to CD33, which is expressed on both normal and malignant myeloid cells and is present on leukemic blasts in over 80% of patients with acute myeloid leukemia (AML) (2). GO was granted accelerated approval by the US FDA in 2000 for the treatment of patients over 60 years of age with relapsed CD33-positive AML (3), but was voluntarily withdrawn in 2010 when a post-approval commitment study failed to confirm clinical benefit and hepatic sinusoidal obstruction syndrome (SOS) became recognized as an adverse event (4, 5, 6). However, several recent clinical trials have clarified the optimal conditions for GO use and demonstrated benefits to specific patient populations (7, 8). Currently, GO is still approved and available in Japan. IO specifically targets the CD22 antigen, which is expressed on the surface of immature and mature B cells and is present in 60-90% of B-lymphoid malignancies (9). IO is currently developed for the treatment of acute lymphoblastic leukemia.

Adverse reactions reported with both these drugs include thrombocytopenia and hepatic injury. Thrombocytopenia with GO, although in part expected from the targeting of CD33-positive hematopoietic progenitor cells, showed prolonged duration in some AML patients who had achieved complete bone marrow remission. For example, persistent thrombocytopenia
(<100,000/µL) was seen in 13% of patients in a Phase II clinical trial with a median time of 66 days to recovery of 50,000 platelets/µL (10). Thrombocytopenia was also one of the main side effects of IO in clinical trials (Grade 3/4 thrombocytopenia in ≥30% of patients) and was one of the most common reasons for dose delay, dose reduction or treatment discontinuation (11). Liver effects reported with GO and IO include increases in aminotransferases, hyperbilirubinemia and hepatic SOS. SOS, previously known as hepatic veno-occlusive disease, is a serious medical condition characterized clinically by jaundice, painful hepatomegaly, weight gain and ascites. SOS is a known complication of hematopoietic stem cell transplantation (HSCT) as a result of the conditioning myeloablative regimen (12). In Phase II trials of GO as a single agent at 9 mg/m²/dose for 2 doses separated by 2 weeks in first relapse AML patients, 9%, 18% and 29% of patients experienced Grade 3 or 4 alanine aminotransferase (ALT), aspartate aminotransferase (AST) and bilirubin elevations, respectively, and SOS was diagnosed in 0.9% of patients in the absence of associated HSCT. In patients who underwent HSCT prior to or after GO treatment, the SOS rates rose to 19% and 15%, respectively (13). In a Phase II trial of IO as a single agent given at a total dose of 1.8 mg/m²/cycle at 4-week intervals for up to 6 cycles in patients with refractory or relapsed ALL, Grade ≥3 hepatic aminotransferase elevations occurred in 6% of patients and SOS was diagnosed in 9% of patients, with 67% of cases observed after post-study HSCT (14).

Similar thrombocytopenia and liver effects were therefore seen in patients with 2 calicheamicin conjugates targeting unrelated antigens and are likely target-independent. Various mechanisms have been postulated for these toxicities, including myelosuppression for platelet effects (1) and target expression on hepatocytes or Kupffer cells for GO liver effects (15, 16). Since the specific
mechanisms are incompletely understood, an investigative study was performed in cynomolgus monkeys with a non-binding ADC containing the same linker and payload as GO and IO with the objectives to further investigate the mechanism for thrombocytopenia development, characterize the liver injury and identify potential biomarkers (hyaluronic acid [HA], plasminogen activator inhibitor-1 [PAI-1] and protein C) for the liver toxicity.
Materials and Methods

Test Article

PF-0259 is an ADC that consists of a non-binding human IgG1 monoclonal antibody linked to the cytotoxic agent N-acetyl gamma-calicheamicin dimethyl hydrazide via the acid-labile 4-(4′-acetylphenoxy) butanoic acid linker. PF-0259 has the same linker and payload as GO and IO. Characterization of PF-0259 was performed by size exclusion chromatography (Acquity UPLC BEH200 SEC, Waters), reverse phase ultra-performance liquid chromatography (Zorbax 300SB-CN, Agilent) and hydrophobic interaction chromatography (TSKgel Butyl-NPR, Tosoh Bioscience). The monoclonal antibody did not bind specifically to any antigen in protein array assays, polyreactivity assays using DNA, insulin and baculovirus particles and showed no binding by immunohistochemistry (IHC) to cynomolgus monkey heart, kidney, liver, lung, skeletal muscle and spleen tissues. The average drug-to-antibody ratio of PF-0259 was 3.9. PF-0259 showed cytotoxic effects comparable to GO and IO in non target-expressing cell assays (eg. average IC50 of 3.0 µg/mL for PF-0259 and GO in CD33-negative Raji cells). The test article PF-0259 was formulated in 20 mM TRIS, 8.5% sucrose, 0.02% polysorbate 80 and 0.005% disodium EDTA (pH: 8.0), diluted in phosphate-buffered saline (PBS), to achieve a final product concentration of 0.5 mg/mL. The vehicle control article consisted of the formulation buffer diluted in PBS.

Animals

Male cynomolgus monkeys (*Macaca fascicularis*) of Mauritius origin, 3 to 4.5 years of age and weighing 3.5 to 6.3 kg at dosing initiation, were obtained from a commercial supplier. All procedures performed on animals in this study were conducted in accordance with established
guidelines and regulations, and were reviewed and approved by Pfizer’s Institutional Animal Care and Use Committee. Pfizer animal care facilities that supported this work are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

**Experimental design**

Male cynomolgus monkeys received up to 3 intravenous bolus administrations 3 weeks apart on Days 1, 22 and 43 of the vehicle control article (N=7) or PF-0259 at 6 mg/m²/dose (N=8) and were necropsied according to protocol randomization on Day 3 (ie. 48 hours after the 1st administration - 1 vehicle control and 2 PF-0259-dosed monkeys) or on Day 63 (ie. 21 days after the 3rd administration - 2 vehicle control and 6 PF-0259-dosed monkeys). A treatment cycle was defined as a single dose followed by a 3-week observation period. The test article PF-0259 and the vehicle control article were administered at a dose volume of 1 mL/kg and the duration of dosing was approximately 1 to 2 min. Four of the 7 monkeys in the vehicle control group were used only for collection of non-terminal parameters, were not necropsied and were returned to the laboratory animal colony at completion of the in-life phase. All animals were evaluated for clinical signs, changes in body weight, food consumption, clinical pathology parameters (including platelet counts, thrombopoietin and interleukin-6 [IL-6]), platelet activation in whole blood, liver microvascular injury biomarkers (HA, PAI-1 and protein C) and trough concentrations of PF-0259 in serum as further described below. Light microscopy, transmission electron microscopy (TEM), histochemistry and/or IHC were performed on selected organs of animals necropsied during the study as detailed below.

**Experimental procedures**
Blood sampling

Blood was collected for hematology in EDTA tubes, for clinical chemistry (including thrombopoietin, IL-6 and HA) in serum separator tubes and for coagulation (including PAI-1 and protein C) in sodium citrate tubes. All parameters were evaluated pre-dose (1 or 2 time points) and on Days 4, 11, 18, 25, 32, 42, 46, 53 and 63. Hematology parameters and thrombopoietin were also evaluated on Days 2, 3 and 7 and IL-6, HA and PAI-1 on Days 2 and 7.

Standard clinical pathology, thrombopoietin and IL-6

Standard hematology parameters were measured using a Siemens Advia 2120 Hematology Analyzer and clinical chemistry parameters using a Siemens Advia 1800 Chemistry Analyzer (Siemens Healthcare Diagnostics, Deerfield, IL). Thrombopoietin and IL-6 were assayed in serum samples by sandwich immunoassays using electrochemiluminescence and flow-based detection, respectively. Thrombopoietin was measured using MSD® Human TPO Kit (Meso Scale Discovery, Rockville, MD) and IL-6 was assayed using Milliplex Non-Human Primate Cytokine Magnetic Bead Panel (EMD Millipore, Merck KGaA, Darmstadt, Germany).

Liver microvascular injury biomarkers (HA, PAI-1 and protein C)

HA was assayed in serum samples using a specific sandwich enzyme-linked immunosorbent assay (ELISA) kit (Echelon Biosciences Inc., Salt Lake City, UT). Citrated plasma was used for the measurement of PAI-1 and protein C. Total PAI-1 concentrations were determined with the Human Serpin E1/PAI-1 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN). Protein C activity was measured by a clotting assay using a STA Compact® Hemostasis Analyzer (Diagnostica Stago Inc., Parsippany, NJ). Briefly, monkey plasma samples were first incubated with a purified extract of Agkistrodon c. contortrix venom for specific activation of protein C,
which leads to inhibition of coagulation factors V and VIII. Protein C activity was then assessed through measurement of APTT in control human plasma samples in which all coagulation factors were present, except protein C, which was derived from the monkey samples being tested.

Platelet activation in whole blood

Platelet activation was evaluated pre-dose and on Days 2 and 18. Whole blood was collected from all animals into CTAD tubes (0.11M sodium citrate with theophylline, adenosine and dipyridamole), which are specifically designed to prevent platelet activation after blood collection. Platelet activation was assessed by analysis of expression of CD61 (Glycoprotein IIb for identification of platelets) and CD62P (P-selectin for assessment of activation) by flow cytometry (FACSCanto, Becton Dickinson, Franklin Lakes, NJ). Positive control samples for platelet activation were generated by adding adenosine diphosphate (ADP) to citrated blood samples from vehicle control animals.

Clinical pathology data evaluation

Individual animal data from the dosing period were compared to respective earliest baseline data, unless otherwise specified. In addition, statistical analysis performed for each parameter at each scheduled sampling time was based on comparisons with concurrent vehicle controls. Each parameter was analyzed either parametrically or nonparametrically using a two-sample test to compare groups. Testing for each parameter was either one-sided for higher values, one-sided for lower values, or two-sided. These choices were prespecified for each parameter. Statistically
significant results were reported in the graphs at the nominal 5%, 1%, 0.5%, or 0.1% level as appropriate.

**Bioanalytical determinations**

Residual serum samples from blood collected from PF-0259-treated monkeys at the end of each cycle (Days 22, 43 and 63) were analyzed for PF-0259 and total antibody concentrations.

**Histopathology and histochemistry**

Animals were fasted overnight before scheduled necropsies on Days 3 and 63. Samples of liver, lung, kidney, heart, bone marrow (sternum) and spleen were fixed in 10% neutral buffered formalin, processed to paraffin blocks and stained with hematoxylin and eosin (H&E) for microscopic examination. In addition, silver stain for reticulin fibers and Masson’s trichrome stain for collagen fibers were performed on liver samples from animals necropsied on Day 63. Bone marrow samples were obtained from flushed femurs and bone marrow smears were stained with May-Grunwald Giemsa.

**Immunohistochemistry (IHC)**

VEGFR2 and CD31 IHC for endothelial cells, CD34 IHC for liver sinusoidal capillarization (17), CD68 IHC for monocytes/macrophages and CD41 IHC for platelets were performed on samples from all necropsied monkeys as detailed in Table 1. All primary antibodies used for IHC were monoclonal antibodies, with the exception of anti-CD41 polyclonal antibody. Unstained 5-micron tissue sections were pretreated for antigen retrieval by heating slides in EDTA for CD31 and CD41 IHC or in Borg® (Tris-based formulation, pH 9.5 - Biocare Medical, Concord, CA) for CD34 IHC using a Decloaking Chamber. Slides were then loaded onto the
Biocare intelliPATH for the staining protocol. Following nonspecific blocking steps, the primary anti-CD31, -CD34 and –CD41 antibodies were applied at dilutions of 1:200, 1:2500 and 1:500, respectively, for 1 hour at room temperature. Detection was performed using Dako EnVision+ System with HRP polymer for amplification and Liquid DAB+ for visualization (Dako, Carpenteria, CA). For CD68 and VEGFR2 IHC, slides were loaded onto the Leica Bond III and pretreated with Epitope Retrieval Solution 2 (Leica Biosystems, Buffalo Grove, IL). The anti-CD68 and the anti-VEGFR2 antibody were applied undiluted or at a dilution of 1:100, respectively, for 15 minutes. Detection was accomplished by using Leica’s Refine Polymer Kit. Isotype control rabbit or mouse IgGs were run on matching tissue sections using similar protocols.

**Transmission electron microscopy (TEM)**

Liver samples from animals necropsied on Day 3 were processed for TEM. Samples were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1M phosphate buffer (Karnovsky’s solution). After post fixation in 1% osmium tetroxide, samples were dehydrated through graded alcohols and propylene oxide prior to embedding in resin. Thick sections were stained with toluidine blue for light microscopy detection of areas of interest. Thin sections of areas of interest were placed on uncoated copper-palladium grids and stained with 10% lead citrate and 2.5% uranyl acetate.
**Results**

**PF-0259 induces acute thrombocytopenia in monkeys**

The general physical condition of all monkeys was normal throughout the experiment and there were no PF-0259-related effects on body weight or food consumption.

Major PF-0259-related hematology changes consisted of decreases in platelet counts (Fig. 1A), with a consistent pattern of acute reversible thrombocytopenia during the 1st cycle and less pronounced but prolonged reductions in platelet counts during subsequent cycles. During the 1st cycle, decreases in platelet counts were observed in all PF-0259-dosed monkeys starting 24 hours (Day 2) after PF-0259 administration (max 0.53x baseline) with nadirs reached at 48 to 72 hours (0.14-0.64x baseline) and complete recovery by the end of the cycle. Subsequent PF-0259 administrations led to milder acute platelet reductions (eg. 0.65-0.75x baseline in 4/6 monkeys 72 hours after the second administration) that did not recover to baseline values over the cycle durations with overall slow downward drifts (0.46-0.80x baseline at the end of the 3rd cycle). Serum thrombopoietin was assessed to further characterize the thrombocytopenia. There were decreases in circulating levels of thrombopoietin in all PF-0259-dosed monkeys, mainly during the 1st cycle from Days 3 to 7 (down to 0.33x baseline) with less pronounced effects seen during subsequent cycles in most animals (Fig. 1B).

Additional PF-0259-related hematology changes of lesser importance consisted of minimal decreases in red blood cell mass from Day 7 and mild to moderate decreases in lymphocyte counts at various time points during the study (data not shown); the reductions in lymphocyte counts were associated with decreased lymphocyte cellularity in the spleen. There were no PF-0259-related effects on other leukocyte populations, including neutrophils.
Trough concentrations of PF-0259 and total antibody in serum at the end of the cycles were overall similar to those observed for a structurally-related ADC (data not shown), indicating adequate systemic exposure and absence of anti-drug antibodies.

**The acute thrombocytopenia induced by PF-0259 is not due to decreased platelet production**

Bone marrow assessment included histological examination of sternal bone marrow and cytological evaluation of femoral bone marrow cytospin smears. There were no PF-0259-related bone marrow histological or cytological alterations in PF-0259-dosed monkeys necropsied on Day 3 or 63, and in particular, the overall hematopoietic cellularity and the megakaryocyte density and morphology were within normal ranges at both necropsy time points (Fig. 1C).

In addition, considering the normal platelet lifespan of 6-8 days in monkeys (18), the rapid decreases in platelet counts (down to 0.53x baseline 24 hours after PF-0259 administration) are consistent with peripheral destruction or sequestration of platelets rather than decreased production by the bone marrow.

**PF-0259 induces acute liver endothelial cell toxicity associated with platelet sequestration in liver sinusoids**

In order to investigate the potential mechanism for the acute thrombocytopenia, selected monkeys were necropsied on Day 3 at the approximate platelet count nadirs.

**Microscopic findings on Day 3 – Liver**

Although there were no remarkable PF-0259-related liver changes at light microscopic examination of H&E-stained slides from monkeys necropsied on Day 3 (Fig. 2A, 2B), IHC for
endothelial cells and platelets demonstrated significant liver alterations in PF-0259-dosed monkeys. As compared with controls, VEGFR2 IHC for endothelial cells showed moderate to marked loss of staining in midzonal and, to a lesser extent, centrilobular regions (Fig. 2C, 2D), which was corroborated by CD31 IHC for endothelial cells (data not shown) and was consistent with loss of sinusoidal endothelial cells (SECs). In addition, CD41 (glycoprotein IIb) IHC for platelets showed moderate to marked granular staining in sinusoids, which was indicative of platelet accumulation, in midzonal regions throughout the sections in PF-0259-dosed monkeys (Fig. 2E-H). There were no appreciable differences in CD68 immunostaining for monocytes/macrophages (including Kupffer cells) between vehicle control and PF-0259-dosed monkeys (data not shown). TEM evaluation of liver samples similarly indicated degeneration and loss of SECs associated with accumulation of numerous platelets in sinusoids, predominantly in midzonal regions (Fig. 3). In addition, sequestered platelets frequently exhibited alterations in cell shape (with formation of surface projections and filopodia in particular) and reduced number or density of cytoplasmic granules, consistent with platelet activation and degranulation, respectively (19). Sinusoids in affected areas often contained sloughed necrotic cells (likely endothelial cells) and scattered leukocytes.

**Microscopic findings on Day 3 – Other organs**

To investigate the liver specificity of the findings reported above, VEGFR2 and CD41 IHC were performed on kidney and lung samples from monkeys necropsied on Day 3 and confirmed the absence of similar changes in these 2 organs (CD41 IHC in Supplementary Fig. S1). CD41 IHC for platelets in spleen showed reduced staining in PF-0259-dosed monkeys as compared with the control monkey, indicating release of platelets from the spleen storage pool secondary to PF-0259-related systemic thrombocytopenia (Supplementary Fig. S2A, S2B).
Platelet activation and cytokine release in systemic circulation

To confirm the lack of direct effects on platelets in the systemic circulation, platelet activation in whole blood and cytokine release (IL-6) in serum were assessed. There were no PF-0259-related increases in activated platelet counts, as assessed by flow cytometry evaluation of platelet CD62P (P-selectin) expression, on Days 2 and 18 in all monkeys when compared with respective individual baseline data. The ADP-stimulated positive control samples showed 33-165x increases in activated platelets when compared to unstimulated control samples, demonstrating validity of the assay (Fig. 1D). There were no PF-0259-related changes in IL-6 throughout the study (Supplementary Fig. S3).

In summary, Day 3 investigations demonstrated PF-0259-mediated liver-specific endothelial injury associated with platelet sequestration in sinusoids, which was considered responsible for the acute thrombocytopenia.

Liver endothelial damage may progress to early SOS changes

To characterize the evolution of liver endothelial damage, microscopic liver findings were characterized in animals necropsied on Day 63 (21 days after the 3rd administration).

PF-0259-related liver findings at light microscopic examination of H&E-stained slides were generally minimal to mild and consisted mainly of multifocal, usually midzonal, sinusoidal dilation and/or hepatocyte atrophy that were best appreciated with a reticulin stain. Sinusoidal dilation was characterized by the presence of small irregular foci of variably dilated sinusoids often filled with red blood cells and separated by thin cords of atrophic hepatocytes. Small foci of hepatocyte atrophy were also observed in the absence of sinusoidal dilation. Silver stain for reticulin fibers further highlighted the finding through the delineation of focally thin
hepatocellular plates (Fig. 4A, 4B). In addition, in areas of sinusoidal dilation or hepatocyte atrophy, Masson’s trichrome stain for collagen fibers showed delicate sinusoidal fibrosis. Other microscopic findings included minimal, multifocal hepatocyte hypertrophy/regeneration and minimal pigment deposition in Kupffer cells in a single PF-0259-dosed monkey.

VEGFR2 and CD34 IHC were performed on liver samples to further demonstrate and characterize endothelial cells. As compared with Day 3 when there was marked loss of VEGFR2 staining, VEGFR2 IHC on Day 63 samples showed only minor and focal alterations in endothelial cell staining in PF-0259-dosed monkeys, mostly in areas of hepatocyte atrophy (Fig. 4C, 4D), indicating significant recovery of the initial endothelial damage. While CD34 IHC in control monkeys only stained endothelial cells associated with or in close proximity of the portal triads and central veins (Fig. 4G), there was mild to marked CD34 immunostaining of SECs throughout the liver lobules in PF-0259-dosed monkeys (Fig. 4H). CD34 overexpression is a marker of sinusoidal capillarization and has been associated with liver functional impairment (17).

These microscopic liver findings in monkeys, including in particular sinusoidal capillarization and sinusoidal dilation/hepatocyte atrophy, were qualitatively similar to those reported in humans after administration of oxaliplatin and were considered to represent early subclinical stages of SOS (17, 20).

Last, there were no appreciable differences in CD41 immunostaining for platelets (Fig. 4E, 4F) and CD68 immunostaining for monocytes/macrophages (including Kupffer cells) in liver samples between control and PF-0259-dosed monkeys on Day 63. Similarly, there were no
appreciable differences in CD41 immunostaining for platelets in spleen samples between control and PF-0259-dosed monkeys on that day (Supplementary Fig. S2C, S2D).

**Hyaluronic acid may be a sensitive mechanism-based diagnostic marker of PF-0259-related liver injury**

**Standard clinical chemistry and coagulation**

PF-0259-related changes in standard liver parameters were limited to overall minimal increases in AST levels (up to 3.09x baseline), with onset usually on Day 4 and sustained effects over the study duration (Fig. 5A). Changes in ALT levels in PF-0259-dosed monkeys were inconsistent. In addition, there were minimal to mild PF-0259-related increases in activated partial thromboplastin time (APTT, up to 1.33x baseline), which were noted mostly at 72 hours after each administration with APTT peaks seen after the 1st dose. Increases in fibrinogen (up to 2.04x baseline), globulin (up to 1.19x baseline) and decreases in albumin (down to 0.84x baseline) were observed in some PF-0259-dosed monkeys at various time points and possibly related to an inflammatory response.

Although AST and APTT increases are likely related to the liver injury, these parameters are not considered suitable diagnostic markers of the liver condition due to the poor liver specificity, narrow dynamic range in this study and/or uncertain mechanism for the changes.

**Exploratory biomarkers**

Following a review of the available literature, potential biomarkers of liver endothelial cell injury measured included PAI-1, protein C and HA (21, 22). There were no PF-0259-related changes in PAI-1 concentration (Fig. 5C) and protein C activity (Fig. 5D) throughout the study. Biomarker
changes were limited to increases in serum HA concentration in all PF-0259-dosed monkeys (Fig. 5B). HA is a polysaccharide present in the extracellular matrix that is synthesized by mesenchymal cells throughout the body and cleared, almost exclusively, by liver SECs (23). Although some fluctuation in serum HA level was seen in control monkeys over the study duration, there were only 2 individual values in control monkeys slightly above 100 ng/mL (both in the same animal). Therefore, individual values in PF-0259-dosed monkeys >100 ng/mL with increases >2-fold the highest respective baseline values were considered to represent an effect of PF-0259. Increases in serum HA concentrations were observed from Day 2 (up to 4.71x baseline), peaked on Day 4 (up to 6.76x baseline) with a trend for recovery over the 1\textsuperscript{st} cycle and sustained increases over subsequent cycles (up to 8.49x baseline). Increases in HA concentrations correlated well with AST levels (R=0.84) and with liver microscopic findings of SEC loss on Day 3 and sinusoidal capillarization and/or sinusoidal dilation/hepatocyte atrophy on Day 63. In particular, the highest HA value (311.9 ng/mL) on Day 63 was recorded in a monkey who had the highest AST increase (3.09x baseline) and liver pathology. The data from this study suggest that elevated serum HA levels in monkeys may be due to decreased clearance by injured hepatic SECs and that HA may be a sensitive mechanism-based diagnostic marker of liver microvascular injury.
Discussion

Thrombocytopenia and hepatic injury including SOS are major adverse events reported in some patients treated with antibody-calicheamicin conjugates such as GO and IO. SOS is a potentially life-threatening liver disorder that is thought to be initiated by injury to SECs (24). We showed that thrombocytopenia and microscopic liver injury consistent with early SOS were similarly seen in monkeys dosed with PF-0259, a non-binding ADC containing the same linker and payload as GO and IO.

The mechanism for thrombocytopenia development was investigated in monkeys. The 3 main mechanisms for a reduced platelet count are impaired production, increased destruction and/or altered distribution (eg. splenic sequestration) (25). The present study suggests that the acute thrombocytopenia observed in PF-0259-dosed monkeys was not due to decreased production of platelets by the bone marrow but resulted from liver sequestration of platelets following liver-specific endothelial injury. The rapid decreases in platelet counts, with onset at 24 hours and up to 86% reduction at 72 hours after PF-0259 administration, were not consistent with an effect on bone marrow production, given the normal platelet lifespan of 6-8 days in monkeys (18). Marked platelet accumulation in sinusoids secondary to SEC degeneration and loss was demonstrated by IHC and TEM in liver samples from monkeys necropsied 48 hours after PF-0259 administration. Similar changes were not present in the lung and kidney from these monkeys, which demonstrated the liver specificity of these alterations.

Our data are consistent with recently published investigations on the mechanism of SOS-associated thrombocytopenia in other clinical settings (26, 27, 28). Liver samples from patients with SOS following oxaliplatin-based chemotherapy or liver transplantation were
immunostained for platelet surface marker CD42b (glycoprotein Ibα) or CD41 (glycoprotein IIb) and demonstrated platelet aggregates or “microthrombi” in sinusoids usually in centrilobular regions. Investigators concluded that platelet sequestration in the liver following SEC damage was the likely mechanism for the observed thrombocytopenias. In addition, the rapid consumption of transfused platelets that is frequently observed in SOS patients (29, 30) is likely due to the same mechanism.

Although liver SEC damage is the initiating event, platelet accumulation and activation within hepatic sinusoids may further exacerbate the liver injury through release of potentially damaging platelet granule contents. Pharmacological inhibition of platelet activation/degranulation might therefore mitigate hepatic injury and SOS in patients. The toxicity of activated platelets to endothelial cells has been shown in vitro and, among the substances released by platelets, thromboxane A2 and serotonin were shown to cause endothelial cell damage while PDGF had no effects (31). Nakanuma, Nishigori and Tajima similarly hypothesized that release of various growth factors by activated platelets (such as thromboxane A2, thrombospondin and VEGF-A) might contribute to the progression of SOS in patients (26, 27, 28). Studies have shown that the anti-VEGF-A antibody, bevacizumab, protects against oxaliplatin-associated SOS (20, 21, 32) and the mechanism, although not completely understood, could involve inhibition of VEGF-A released from activated platelets. Recently, Cilostazol, a phosphodiesterase 3 inhibitor with antiplatelet properties, was reported to prevent SOS following living donor liver transplantation (33).

Although the mechanism for the acute thrombocytopenia in monkeys has been elucidated, the cause for the lack of complete platelet recovery following repeated administrations of PF-0259 remained incompletely understood, given the absence of bone marrow damage or of significant
platelet sequestration in the liver on Day 63. As thrombopoietin is mainly produced by the liver (34), the observation of decreases in serum thrombopoietin might be related to the ongoing liver alterations and indicative of inadequate bone marrow stimulation with concomitant decreased ability to achieve normal platelet numbers.

Microscopic liver findings in monkeys after repeated administrations of PF-0259 included sinusoidal capillarization and sinusoidal dilation/hepatocyte atrophy, which have been described in patients with SOS (17, 20). As compared to Day 3, there was significant recovery of the initial endothelial cell loss with CD34 overexpression by recovered cells. CD34 expression is a marker of sinusoidal capillarization, which is a pathological finding characterized by alterations of endothelial cells (ie. development of a basement membrane and loss of fenestrations) and liver functional impairment (17, 35). In addition, the observation of CD34 overexpression suggests recovery of endothelial damage through recruitment of bone marrow-derived CD34-positive endothelial progenitor cells, as has been shown in other vascular injury settings (36). Recently, infusion of endothelial progenitor cells was shown to mitigate liver injury in mice after HSCT (37).

Another objective of our work was to evaluate biomarkers of liver injury. There are currently no validated blood tests for SOS and the diagnosis is mainly based on clinical and laboratory criteria that are nonspecific and/or late events in the development of the disease (12). SOS-associated endothelial injury triggers a hypercoagulable state and several studies have evaluated in human patients the diagnostic value of proteins involved in coagulation, such as PAI-1 and protein C (22, 38), with inconsistent results. PAI-1 is an inhibitor of fibrinolysis that is synthetized and released by endothelial cells and protein C has anticoagulant activity through inactivation of Factors Va and VIIIa. There were no PF-0259-related changes in PAI-1 and protein C in any
monkey throughout the study and these parameters were not sensitive markers of endothelial
damage in these experimental conditions. HA is a polysaccharide present in the extracellular
matrix that is synthesized by mesenchymal cells and cleared, almost exclusively, by liver SECs 
(23). Systemic HA levels can be increased with liver SEC structural and/or functional damage
and have been explored clinically for the noninvasive detection of SOS with promising results
(21, 39). Evaluation of HA levels in monkeys showed early and sustained increases in HA that
correlated well with AST levels and liver pathology on both Days 3 and 63, indicating the ability
of this parameter to detect both early structural damage (Day 3) and later functional impairment
(Day 63) of SECs. HA is however not a specific marker of liver SEC damage since there are
other mechanisms that can lead to increased serum levels, such as increased HA production
during the course of fibroplasia (40, 41). Despite the limitations related to the lack of HA
specificity, our data provide additional support to the diagnostic value of HA for the sensitive
detection of liver microvascular injury, which should be explored in future work for confirming
its potential as a biomarker in humans.

Lastly, we hypothesize that the mechanisms demonstrated in monkeys for the target-independent
liver injury and thrombocytopenia of antibody-calicheamicin conjugates may operate for other
classes of ADCs. Trastuzumab emtansine (T-DM1) is an ADC comprised of a microtubule
inhibitor conjugated via a stable linker to a humanized monoclonal antibody against human
epidermal growth factor receptor 2 (HER2). Major adverse events in patients include
thrombocytopenia and liver effects. The thrombocytopenia is usually characterized by: 1) acute
onset with decreases in platelet counts as soon as 1 day after T-DM1 administration (42), 2)
platelet nadirs during cycle 1 (3-week cycle) with recovery by end of cycle, and 3) slow
downward drifts in platelet counts in some patients over multiple T-DM1 cycles (43). In
addition, it is noteworthy that hematological effects with T-DM1 are quite selective for platelets and other hematological lineages are relatively unaffected. Liver effects with T-DM1 are characterized by increases in serum aminotransferases along with occasional cases of nodular regenerative hyperplasia (NRH) (44). NRH is a liver microvascular disorder that, similar to SOS, is thought to result from an initial insult to liver SECs. Interestingly, conditions associated with SOS development, such as HSCT procedure and oxaliplatin treatment, are also associated with NRH development, suggesting a pathogenetic link between SOS and NRH. The overall similarity of effects on platelets and liver with T-DM1 and antibody-calicheamicin conjugates suggests that a similar underlying mechanism may contribute to the thrombocytopenia and liver injury with both classes of drugs. However, these similarities require further investigation in light of another recently proposed hypothesis for T-DM1-mediated liver injury, namely HER2 expression by hepatocytes (45).

In conclusion, this work has investigated the liver toxicity and thrombocytopenia of antibody-calicheamicin conjugates and suggested a relationship in monkeys between these 2 pathological processes, through target-independent damage to liver SECs that is associated with platelet sequestration in liver sinusoids. We further hypothesize that a similar mechanism may operate for other ADCs in patients where adverse events of thrombocytopenia, increased liver enzymes and liver microvascular disorders (including NRH) have been observed.
References


Proceedings of the 56th Annual Meeting of the American Society of Hematology (ASH); 2014 Dec 6-9; San Francisco, CA. Abstract nr 2255.


Figure Legends

Figure 1 – PF-0259 induced thrombocytopenia in monkeys in the absence of myelosuppression or of direct effects on platelets in systemic circulation. Monkeys were dosed intravenously with vehicle or PF-0259 at 6 mg/m²/dose once every 3 weeks on Days 1, 22 and 43. A, effects of PF-0259 on blood platelet counts. PF-0259 induced acute reversible thrombocytopenia during the 1st cycle and less pronounced but prolonged decreases in platelet counts during subsequent cycles. B, evaluation of serum thrombopoietin concentration did not demonstrate upregulation of this hormone in association with thrombocytopenia. C, histology of sternal bone marrow on Day 3 and Day 63. There were no PF-0259-related alterations in hematopoietic cellularity and megakaryocyte density and morphology at both necropsy time points. H&E stain, scale bar = 60 µm. D, evaluation of platelet activation in whole blood by flow cytometry analysis of expression of CD61 (Glycoprotein IIb for identification of platelets) and CD62P (P-selectin for evaluation of activation). Positive control samples were generated by adding adenosine diphosphate (ADP) to blood samples from vehicle control animals. PF-0259 did not induce platelet activation in systemic circulation on Days 2 and 18. Data in A, B and D are represented as the mean values ± 1 standard deviation (SD). Significantly different from vehicle control: * - p ≤ 0.05, † - p ≤ 0.01, ‡ - p ≤ 0.005, § - p ≤ 0.001.

Figure 2 – PF-0259 induced acute liver sinusoidal endothelial cell injury associated with intrasinusoidal platelet sequestration. Monkeys were necropsied 48 hours after a single intravenous administration of vehicle or PF-0259 at 6 mg/m². Light microscopic evaluation of
liver from vehicle control (A, C, E, G) and PF-0259-dosed (B, D, F, H) monkeys. There were no remarkable PF-0259-related liver changes at light microscopic examination of H&E-stained slides (A, B). VEGFR2 IHC showed delicate and diffuse staining of sinusoidal endothelial lining cells in control monkey (C) while there was staining disruption and marked loss of VEGFR2 immunoreactivity in midzonal and to a lesser extent centrilobular regions in PF-0259-dosed monkey (D). CD41 IHC for platelets showed minimal scattered punctate staining in vascular spaces in control monkey (E, G) while there was abundant intrasinusoidal granular staining in midzonal regions throughout the liver sections in PF-0259-dosed monkey, consistent with platelet accumulation (F, H). The lower magnification (F) demonstrated the midzonal distribution and the higher magnification the intrasinusoidal location (H) of the staining. pa, portal area; cv, central vein. Scale bar = 100 µm.

**Figure 3** – Electron microscopic (EM) evaluation of liver samples confirmed PF-0259-related acute midzonal sinusoidal endothelial cell (SEC) injury associated with platelet sequestration. Monkeys were necropsied 48 hours after a single intravenous administration of vehicle or PF-0259 at 6 mg/m². Transmission EM evaluation of liver from vehicle control (A) and PF-0259-dosed (B) monkeys. A, note the normal aspect of the fenestrated endothelium (arrows) lining the space of Disse in the control monkey. Asterisk *, lumen of sinusoid; H, hepatocyte; RBC, red blood cell. B, There was loss of sinusoidal endothelial lining cells with direct contact between the space of Disse and the lumen of the sinusoids (black arrows) in the PF-0259-dosed monkey. The endothelial cell loss was associated with intrasinusoidal accumulation of platelets (red arrows) showing alterations in cell shapes such as surface projections. There were also scattered sloughed necrotic cells (likely endothelial cells) and leukocytes in the lumen of the sinusoids. Scale bar = 1 µm.
Figure 4 – Liver sinusoidal endothelial damage progressed to microscopic changes consistent with early stages of sinusoidal obstruction syndrome (SOS). Monkeys were dosed intravenously with vehicle or PF-0259 at 6 mg/m²/dose once every 3 weeks and were necropsied on Day 63 at the end of the 3rd cycle. Light microscopic evaluation of liver from vehicle control (A, C, E, G) and PF-0259-dosed (B, D, F, H) monkeys. The reticulin stain showed regularly-sized hepatocellular plates in the control monkey (A) while it clearly demonstrated scattered, usually midzonal, foci of hepatocellular atrophy in PF-0259-dosed monkey (B). VEGFR2 IHC for endothelial cells (C, D) showed focal alteration and loss of VEGFR2 staining in areas of hepatocellular atrophy in PF-0259-dosed monkey (D), consistent with focal persistent loss of sinusoidal endothelial cells (SECs). CD41 IHC for platelets (E, F) did not demonstrate any significant sequestration of platelets in liver sinusoids in PF-0259-dosed monkey at this stage (F). CD34 IHC only stained endothelial cells associated with or in close proximity of the portal triads and central veins in the control monkey (G) while there was moderate to marked CD34 immunostaining of SECs throughout the liver lobules (outside of areas of hepatocellular atrophy) in the PF-0259-dosed monkey, which is indicative of sinusoidal capillarization (H). pa, portal area; cv, central vein. Scale bar = 100 µm.

Figure 5 – Effects of PF-0259 on potential circulating markers of liver injury. Monkeys were dosed intravenously with vehicle or PF-0259 at 6 mg/m²/dose once every 3 weeks. There were early and sustained increases in hyaluronic acid (HA; up to 8.5x) (B) that correlated well with AST levels (A) and liver pathology on both Days 3 and 63. There were no PF-0259-related changes in plasma plasminogen activator inhibitor-1 (PAI-1) concentration (C) and protein C activity (D) throughout the study. Data are represented as the mean values ± 1 standard deviation.
(SD). Significantly different from vehicle control: * - p ≤ 0.05, † - p ≤ 0.01, ‡ - p ≤ 0.005, § - p ≤ 0.001.
<table>
<thead>
<tr>
<th>Molecular target</th>
<th>Antibody clone</th>
<th>Samples evaluated</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Day 3</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>55B11 (Cell Signaling Technology, Danvers, MA)</td>
<td>Liver, Kidney, Lung</td>
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<td>CD68</td>
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<tr>
<td>CD41</td>
<td>Polyclonal (Sigma-Aldrich, St Louis, MO)</td>
<td>Liver, Kidney, Lung, Spleen</td>
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</table>
Disclosure of Potential Conflicts of Interest

All authors are employees and/or shareholders of Pfizer, Inc., a publicly traded company.

Authors' Contributions


Study supervision: K. Walters

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Acknowledgements

The authors thank Kiran Khandke for designing and producing the antibody-calicheamicin conjugate used in this study, Walter Bobrowski and Germaine Boucher for excellent assistance with the photomicrographs and Sarah Lopes for quality control of the manuscript.
Grant Support

This work was supported by Pfizer Inc.
Figure 2

Vehicle Day 3

PF-0259 Day 3

H&E

VEGFR2 IHC

CD41 IHC

G

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Clin Cancer Res  Published OnlineFirst September 28, 2016.

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