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Drug-induced thrombocytopenia: mechanisms and relevance in preclinical safety assessment

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Abstract

Thrombocytopenia is one of the most commonly observed drug-induced adverse hematologic toxicities in the clinic. Therefore, *in vitro* and *in vivo* evaluations of effect of drugs on platelets are an important component in preclinical safety assessment in drug development. To date, a number of mechanisms have been identified to be associated with drug-mediated thrombocytopenia. Amongst these, some are conserved across species whereas others are not. Therefore, a case-by-case approach is needed to assess drug-induced thrombocytopenia at preclinical stages to understand translatability to humans. The present chapter reviews mechanisms in drug-mediated thrombocytopenia with a focus on nonimmune (direct myelotoxicity) as well as immune-mediated thrombocytopenia by both small and large molecule therapeutics. Several *in vitro* and *in vivo* models as well as challenges in assessing drug-mediated thrombocytopenia in preclinical stages will also be discussed.

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Platelets, Drug-induced thrombocytopenia, Toxicology, Safety assessment.

Introduction

Platelets are anucleate, peripheral blood cells that play a fundamental role in thrombosis and hemostasis. Produced by megakaryocytes in bone marrow (also called thrombopoiesis), platelets are terminally differentiated blood cells that circulate in blood with an estimated lifespan of 7–10 days. At the site of vascular injury, platelets are consumed by clot formation to stop bleeding. Alternatively, aging platelets are removed primarily by splenic macrophages and hepatocytes [58]. Recent studies showed clearance of aging platelets often involved phosphatidylserine exposure and/or deglycosylation on platelet plasma membrane that can be recognized by macrophages and hepatocytes [58]. Under certain pathological conditions such as immune thrombocytopenia (ITP), healthy platelets can be opsonized by antiplatelet antibodies which results in an Fc receptor–mediated clearance by macrophages [88].

Thrombocytopenia is a potential drug side effect commonly observed in humans and animals [3,68]. For instance, chemotherapeutic agents induce thrombocytopenia by suppressing the development and/or differentiation of megakaryocytes or hematopoietic progenitors. Several other drugs also mediate development of drug-dependent antiplatelet antibodies (i.e., drug-induced ITP, DITP) that can lead to an increased clearance of platelets. The intent of this article is to review the most commonly identified mechanisms for drug-induced thrombocytopenia. *In vitro* and *in vivo* models as well as challenges in assessing drug-induced thrombocytopenia will also be discussed.

Mechanisms of drug-induced thrombocytopenia

Myelosuppression

As the lifespan of platelets is relatively short (7–10 days), a healthy and sufficient megakaryocyte population is required to maintain a stable platelet count in blood [32]. Designed to target fast dividing cancer cells, chemotherapeutics including cytotoxic agents, alkylating agents, and antimetabolites, often inhibit mitotically active bone marrow precursor cells including megakaryocytes which can lead to thrombocytopenia by decreasing platelet production [14,39]. A retrospective evaluation of over 200,000 cancer patients, in a recent study showed that around 10% patients developed chemotherapy-induced thrombocytopenia, which continues to be a concern during cancer treatment [84].

Myelosuppression/toxicity is also one of the most commonly observed adverse effects in patients treated with small molecule tyrosine and serine/threonine kinase inhibitors (KIs), a newer generation of targeted anticancer therapies [6,33]. Many KIs, including those targeting epidermal growth factor receptor pathway (e.g., gefitinib and erlotinib), vascular endothelial growth factor pathway

(e.g., sorafenib and sunitinib), and BCR-ABL pathway (e.g., imatinib and dasatinib), have been reported to induce thrombocytopenia in patients [5,6,55,59,72]. Mechanisms of KI-induced thrombocytopenia are most commonly associated with the inhibition of signal transduction pathways that are critical for the growth and differentiation of bone marrow stem cells and/or progenitor cells including megakaryocytes [27]. For instance, receptor tyrosine kinase Kit (c-Kit) and fms-like tyrosine kinase 3 (FLT3) are important regulators of hematopoiesis [46]. Not surprisingly, administration of KIs with potent c-Kit antagonism or c-Kit/FLT3 dual antagonism can result in profound myelosuppression [71]. It has also been demonstrated that relative differences in potency against c-Kit and FLT3 by various KIs may also contribute to the variable hematological effects observed in the clinic, for example, sunitinib tended to cause neutropenia although pazopanib tended to cause thrombocytopenia [38,81].

Antibody–drug conjugates (ADCs), where antibodies are coupled with cytotoxic drugs (e.g., maytansine), cause thrombocytopenia because of bone marrow toxicity particularly targeting megakaryocyte lineage [23,25]. Designed to selectively deliver cytotoxic payload to tumor cells, ADCs are developed to minimize systemic toxicity. However, some toxicities, including thrombocytopenia, were still observed in humans when dosed with some ADCs including T-DM1 (KADCYLA®, a human IgG1 anti-HER2 monoclonal antibody (mAb) conjugated with maytansine derivative DM1), and AGS-16C3F (a fully human anti-ectonucleotide pyrophosphatase/phosphodiesterase (ENPPs) IgG2 monoclonal conjugated to monomethyl auristatin F, currently at phase II clinical trial) [74,75]. Mechanisms of ADC-mediated thrombocytopenia is attributable to the inhibition of megakaryocyte differentiation and maturation [74,75,77]. Several studies showed that FcγRIIa or macropinocytosis-mediated internalization of ADCs likely contribute to a higher drug exposure in megakaryocytes than in other tissues, which could explain the high incidences of thrombocytopenia in the clinic (ranging from >10% to >50% in various clinical trials) [77,87].

Myelosuppression of anticancer drugs generally affect multiple hematopoietic lineages. However in some instances, megakaryocyte lineage is the only one impacted, especially, when treated with targeted anticancer drugs such as KI and ADCs. In addition, as it takes some time (~5 days in human) for megakaryocytes to mature and release platelets, thrombocytopenia mediated by myelosuppressors may show a delayed onset (~1–2 weeks post treatment) [14,44]. Most anticancer drug-mediated thrombocytopenia is dose-dependent and generally reversed after withdrawal of the treatment [14,36].

Besides anticancer therapies, few drugs for other indications have been reported to induce a similar general myelosuppression effect, likely due to the relative low tolerance to myelosuppression in those therapeutic areas. Interferon alpha (IFN-α), a chronic hepatitis C therapy, that has been reported to mediate a mild (~20% patients) to severe (~1% patients) thrombocytopenia in the clinic. Multiple mechanisms may contribute to IFN-α–mediated thrombocytopenia in the clinic [26,69]. Several studies showed that inhibiting platelet production by inhibiting megakaryocytes function can play important roles in IFN-α–mediated thrombocytopenia [30,85].

Immune-mediated mechanisms

Thrombocytopenia can also be induced by drugs that accelerate platelet destruction, which most commonly involves immune-mediated mechanisms characterized by the development of antiplatelet antibodies [4,3,36,48]. Platelet-bound antibodies can mediate various effector functions including antibody-dependent cellular cytotoxicity, antibody-dependent cellular phagocytosis, and complement-dependent cytotoxicity which result in the expedited clearance of platelets [4,3].

Previously, hundreds of small molecule and biologic drugs or drug candidates in various classes, including (but not limited to) antibiotics, antiviral, anticoagulant, antiarrhythmic, anticonvulsant, antidiabetic, and antirheumatic, have been reported to carry the risk of inducing ITP in human. In many cases, DITP showed an idiosyncratic feature, that is, only affect a small fraction of patients who took the medications [4,3].

Several well-known mechanisms of DITP are summarized in the following section.

Hapten formation

One of the most well-documented mechanisms is the drug–hapten formation. Haptens are defined as nonimmunogenic small molecules (molecular weight typically less than 2–5 kDa) that are not capable of inducing an immune response by themselves, but can mount an immune response when covalently attached to a carrier protein [15]. For instance, the beta-lactam ring in penicillin derivatives, have been shown to covalently bind to epsilon-amino groups of lysine residues of proteins through the penicilloyl group after the opening of beta-lactam [40,43,83]. Covalent binding of penicillin to platelet membrane proteins results in the formation of a new antigenic epitope (i.e., neoepitope) that can trigger an immune response and lead to antihapten antibody formation. Upon re-exposure of a sensitized individual to penicillin, reforming of drug–platelet complex providing a target for antihapten antibodies and enabling it to cause platelet destruction [4,40,43].

Another example of thrombocytopenia mediated by drug–hapten formation is heparin. Heparin can mediate

both as nonimmune-mediated and an immune-mediated thrombocytopenia. Upon receiving the drug, about 25% of patients develop a low grade, rarely symptomatic thrombocytopenia attributable to increased platelet aggregation because of prolonged adenylate cyclase inhibition (nonimmune-mediated, type I heparin-induced thrombocytopenia, HIT) [16,17]. However, a subset of patients (~1%) can develop a moderate to severe thrombocytopenia with concomitant venous and/or arterial thrombosis, which can be life-threatening (immune-mediated, type II HIT) [1,17]. Type II HIT is caused by antibodies that recognize complexes formed by heparin and platelet factor 4 (PF4), a 32-kDa CXC chemokine found in platelet alpha-granules. Heparin binding to membrane bound PF4, a positively charged chemokine, resulted in the formation of a neoantigen which trigged an antibody response [31,60]. In addition, antihapten antibodies can bind to soluble PF4 via their Fab portion to form immune complexes which can activate platelets via Fc binding to platelet FcγIIa receptors [35,78]. The combined effects resulted in a symptom in the clinic showing thrombocytopenia accompanied by a prothrombotic state [35,78,79].

Drug-mediated noncovalent binding to glycoprotein

Drug-dependent antiplatelet antibody can also be induced by epitope formation by drugs noncovalently binding to platelet membrane glycoproteins (GPs, usually GPIIb/IIIa and/or GPIb/V/IX) [4,9,10]. In this scenario, bound drugs are in the soluble form and do not act as a classical hapten. Quinine drugs and some sulfonamide antibiotics have been shown to induce antiplatelet antibodies by this mechanism [4,9,10].

Drug-mediated conformational change in GPIIb–IIIa

Platelet GPIIb–IIIa inhibitors represent an alternative mechanism to induce antiplatelet antibodies. Fibans, including tirofiban and eptifibatide, are synthetic agents that bind tightly to the Arg-Gly-Asp (RGD) motif in GPIIb–IIIa. Abciximab is a Fab fragment of a humanized mouse chimeric mAb against GPIIb/IIIa. These drugs prevent formation of platelet thrombi by blocking reaction of activated integrin with fibrinogen and other ligands, and are widely used to reduce complications following percutaneous transluminal coronary angioplasty [18,24,65]. Binding of these drugs caused confirmational changes to GPIIb/IIIa, which were recognized by either antidrug antibodies or pre-existing antibodies resulting in an acute, often severe, thrombocytopenia in some patients (~1–2%) [3,11,29]. Abciximab can also induce antidrug antibodies against its murine sequences which can also contribute to thrombocytopenia upon repeated exposure to the drug.

Drug-induced autoantibody

In rare cases, some small molecule drugs can also trigger a drug-independent, platelet-specific autoantibody response, a phenotype mimicking chronic autoimmune thrombocytopenic purpura (AITP). Platelet-specific autoantibodies similar to AITP occurred in 1–2% of patients treated with gold salts for rheumatoid arthritis [80]. Other medications implicated as possible triggers for AITP include L-dopa, procainamide, penicillamine, and sulfamethoxazole [3,36,79,80].

A number of mAb drugs, including adalimumab (anti-TNFα, v™), efalizumab (anti-CD11a, Raptiva™), and alemtuzumab (anti-CD52, Campath™), induce thrombocytopenia in a small proportion of patients (typically ranging from <1% to 5% depending on the drug and patient population) [13,21,29,82]. Thrombocytopenia, in these instances, can be resolved in most patients by corticosteroids and/or intravenous immunoglobulin, suggesting pre-existing or de novo generation of anti-platelet antibodies may contribute to thrombocytopenia observed in these patients. The mechanism of autoantibody development by these monoclonal antibodies are either unclear or suspected to be associated to the pharmacological effects of these drugs [29].

Other mechanisms

Besides myelosuppression and immune-mediated mechanisms, a few other mechanisms can lead to drug-induced thrombocytopenia. For instance, a few drugs, including cisplatin, balhimycin, aspirin, and vancomycin, have been shown to enhance platelet clearance by inducing platelet apoptosis [76]. Other drugs can induce thrombocytopenia due to secondary drug effects on other tissues and cells, such as immune cells and endothelium. For instance, several biotherapeutics, including TGN-1412, OKT3 (Muromonab), anti-IL6 (Tocilizumab) and chimeric antigen receptor (CAR) T-cell therapies (Kymriah and Yescarta), have been reported to develop mild to severe thrombocytopenia [12,37,50,73]. There is no evidence that these drugs can bind directly to platelets. Platelet decrease by these drugs is likely secondary to an acute inflammatory response mediated by drug-induced immune activation.

Preclinical safety assessment for drug-induced thrombocytopenia

Before testing in clinical trials, pharmaceutical products are required to undergo rigorous preclinical safety assessment in preclinical species including but not limited to mouse, rat, dog, and\or nonhuman primates.

Like other drug-induced organ toxicities, drugs that induce thrombocytopenia in animal studies can be very helpful for not only understanding translatability of this clinically monitorable finding to humans, and thereby

calculation of safety margins relative to estimated human exposure, but also reversibility and potential mechanisms of thrombocytopenia across species. Thrombocytopenia mediated by myelosuppressive agents are often conserved across species. Classical chemotherapy drugs (such as cisplatin, 5-fluorouracil, doxorubicin, and cyclophosphamide), KIs (such as imatinib, dasatinib, and sunitinib), ADCs (such as including T-DM1 and AGS-16C3F), and a relatively recent example selinexor, an oral selective nuclear export inhibitor, have all been shown to induce thrombocytopenia in one or more animal species [41,45,47,49,53,56,57,86]. In this scenario, preclinical animal studies often provide critical toxicological parameters such as maximum tolerated dose and no observed adverse effect level that can potentially guide dose selection in clinical trials. Besides animal studies, the megakaryocyte-based colony-forming assay (MK-CFC), an *in vitro* hematopoietic stem cell or progenitor cell—based proliferation assay, has also been widely used to assess effects of myelosuppressive drugs [54,63]. The assay was originally developed in 1980s by culturing bone marrow cells in methylcellulose in the presence of thrombopoietin, a key growth factor that regulates megakaryocyte differentiation and platelet production. Later on, a modified cell proliferation—based liquid-phase platform was developed to increase assay throughput [54,63]. Retrospective studies using known myelosuppressive agents showed that drug potency in the *in vitro* colony-forming assay often correlated to the clinical dose showing hematopoietic suppression, suggesting the potential predictive values of using these assays to assess thrombocytopenia risk early on in drug development [54,64].

In contrast to myelosuppressant-mediated thrombocytopenia, most DITP, especially those induced by small molecule drugs, are not conserved across species with some rare exceptions such as gold salt have been reported to induce thrombocytopenia in dogs [7]. The idiosyncratic feature and no clear dose—response relationship of DITP often makes the toxicity challenging to be assessed in routine preclinical animal studies. In some cases, engineered animal models can be helpful to understand mechanisms of DITP. For instance, Reilly et al. [62,61] developed transgenic mice that expressed either human FcγRIIA alone, or human PF4 alone, or both human FcγRIIa and PF4. By injecting a human antiheparin antibody in control or transgenic mice, the team identified thrombocytopenia was only apparent in FcγRIIa/PF4 double transgenic mice but not in single knockout or control animals, suggesting both FcγRIIa and PF4 are required for antiplatelet antibody-mediated thrombocytopenia [62,61]. Few *in vitro* cell-based models can predict DITP as well. *In vitro* assessment for DITP has been primarily focusing on developing tools to detect antiplatelet antibodies in serum or plasma samples collected from patients previously exposed with drugs suspected of inducing thrombocytopenia. These methods include the use of radiolabeled or fluorescein-labeled antihuman immunoglobulin (Ig) secondary antibodies to detect platelet-bound Ig's by enzyme-linked immunospecific assay (ELISA), flow cytometry, or immunoprecipitation-Western blotting (IP-WB) [2,4]. In the past several years, flow cytometry has been increasing used as a rapid and highly sensitive technique to detect drug-dependent antiplatelet antibodies in both human and animals. For human samples, a typical flow cytometry-based assay involves incubation of serum or plasma samples with group O platelets in a microtiter plate with or without the presence of a drug or a drug metabolite suspected to induce an antiplatelet antibody response [2,4,22]. The concentration of a drug used in the assay are often comparable to *in vivo* exposure, e.g., close to maximum (or peak) serum concentration (Cmax). After washing, platelets are then incubated with a fluorescent-labeled antihumanIg) secondary antibody for the detection of bounded antiplatelet antibodies (IgG and/or IgM). Platelets can be identified by a flow cytometer based on their size and surface granularity (forward angel scatter vs side angel scatter). And the amount of antiplatelet antibodies are quantified by the fluorescent intensity detected in single platelets detected by the flow cytometer [2,22].

Dose-dependent changes in platelet counts have been observed after treatment in cynomolgus monkeys for some antisense oligonucleotides (ASOs) that contain phosphorothioate in the backbone linkage and/or 2′-O-methoxyethyl (2′-MOE) in the sugar moiety [34]. Animals presenting with this phenotype have platelet counts that are reduced, from baseline, by approximately 30%—50% in a dose-dependent fashion but achieve a new "steady-state" level that generally remains within the normal range with continued treatment (≥150 K cells/μl). This phenotype has been observed in monkeys with approximately 40% of evaluated 2′-MOE ASOs and thus appears to be sequence-specific. Decreases in platelet count of this nature have occasionally been observed in clinical trials with 2′-MOE ASOs [20], with 3 of 16 2′-MOE ASOs exhibiting phenotype similar to this in humans. Unlike DITP induced by generation of antiplatelet IgG antibodies, mechanism by which this occurs is associated with an increase in natural IgM, in concert with monocyte activation, leading to increased platelet sequestration in the spleen and liver, and ultimately reduced platelet counts in peripheral blood [51]. Enhanced valency of IgM (n = 10), despite their low antigen-binding affinities, can facilitate direct interaction with functional IgM clearance receptors and/or complement receptor [42], on macrophages thereby enhancing the phagocytic capacity of macrophages [52,67,70]. Even though, ISIS 104838-induced complement activation is not only a Cmax-dependent effect but also occurs proximal to platelet (PLT) decrease, it is tempting to speculate that

deposition of C3 along with IgM antibodies, a more efficient activator of complement than IgG, on the PLT surface can lead to PLT opsonization, followed by increased clearance of PLTs in the spleen and liver [8]. Even though, a notable exception, severe sporadic thrombocytopenia (platelet counts <50 K cells/μL) also has been observed at higher dose levels in toxicology studies for some 2′-MOE ASO and occurs most often in one to three cynomolgus monkeys in a dose group and can be precipitated by pre-existing antiplatelet IgG antibodies [51]. These dose-dependent sporadic decreases have not been translated to humans based on the relative absence of similar severe changes in humans across multiple 2′-MOE ASO clinical programs [20,34]. An apparent exception to this experience has been the very recent observation of severe thrombocytopenia in patients in two placebo-controlled phase III trials in two rare disease populations, in which two different 2′-MOE ASOs, inotersen and volanesorsen, were given to patients with mutant transthyretin amyloidosis and familial chylomicronemia syndrome (FCS), respectively. The low incidence of severe thrombocytopenia, whether in nonhuman primates or in humans, suggests some contributing factor(s) that influence individual susceptibility, either due to the underlying disease or some other condition that sensitizes the platelet to increased clearance. Although this is still an active area of investigation, in patients with FCS , it has recently been reported that substantial variations in platelet counts over time, including platelet counts <40 K cells/mL, have been shown to be a part of the natural history of the disease [19].

In some rare cases, DITP can occur in animals that may not be translatable to human. Two monoclonal antibody drug candidates, AMGX (anti−human soluble protein) and LY2541546 (antisclerostin), have been shown to directly bind to platelets through an off-target mechanism in animals (cynomolgus monkeys for AMGX, and rats for LY2541546) [66,68]. AMGX and LY2541546 mediated a dose-dependent decrease in platelets in cynomolgus monkeys and rats, respectively. *In vitro* analysis showed that AMGX did not bind to human platelets, although LY2541546 did not bind to either cynomolgus monkey or human platelets, suggesting in both cases antibody mediated off-target binding are species specific [66,68]. Another case is mAbY.1, a fully human IgG2 mAb biotherapeutic against a human cell surface protein known to be absent in platelets [28]. mAbY.1 caused a dose-depending decrease of platelets within 6 h after intravenous administration. mAb.1 did not bind to cynomolgus peripheral blood or bone marrow cells. Mechanism of mAbY.1-mediated thrombocytopenia has been shown to directly or indirectly activate splenic macrophage, as an increased platelet phagocytosis was detected in an *in vitro* monocyte/platelet co-culture assay. The effect has been shown to be cynomolgus-specific as mAbY.1 did not enhance human platelet phagocytosis in a similar assay [28]. These cases showed that when thrombocytopenia is observed in animals, understanding the mechanism of toxicity and developing translation assays if feasible can be helpful to assess the relevance of animal findings to human risk.

Conclusion

Drug-induced thrombocytopenia is a commonly observed adverse effect in the clinic. Various mechanisms, including myelosuppression, hapten formation, noncovalent binding, protein structural changes, induction of autoantibodies, platelet apoptosis, and immune activation, have been demonstrated to contribute to the pathogenesis of drug-induced thrombocytopenia. Drug-induced myelosuppression and platelet apoptosis are often conserved cross-species and can be evaluated *in vivo* (animals) as well as *in vitro* (MK-CFC) at the preclinical drug development stages. In contrast, drug-induced ITP are often challenging to be modeled at the preclinical stages due to the idiosyncratic feature and species differences. Therefore, the field has been mainly focused on the development of antiplatelet antibody measurements to enable the diagnosis of DITP. In summary, past experiences suggested that a case-by-case based, weight of evidence approach with a holistic analysis of various evidences including drug modality, intended target, off-target profiles, as well as findings in animals and human will be important for assessing the risk of drug-induced thrombocytopenia.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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