Emerging treatments for thrombocytopenia: Increasing platelet production

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Thrombocytopenia is a common medical problem. The first generation thrombopoietic agents, recombinant THPO and 'megakaryocyte growth and development factor' (PEG-rHuMGDF) entered clinical trials, but their development was discontinued owing to neutralizing auto-antibodies cross-reacting with endogenous THPO, causing thrombocytopenia in healthy volunteers. Although an approved drug for prevention of severe thrombocytopenia following myelosuppressive chemotherapy (human Interleukin-11) exists, the search for new thrombopoietic agents continued because its use is limited by side effects. Several second generation thrombopoietic factors have entered clinical trials and some new negative regulators of megakaryopoiesis have been found, such as platelet factor 4 (PF4) and the pituitary adenylate cyclase activating polypeptide (PACAP). Their inhibition may be useful in the treatment of thrombocytopenia. This article reviews second generation thrombopoietic factors and those recently discovered regulators of megakaryopoiesis.

Introduction

Thrombocytopenia, defined as a subnormal number of platelets (usually below 150×10^9/L) in the circulating blood, is a common problem in the management of patients with cancer and other conditions that affect hematopoietic cells. Mild thrombocytopenia is defined as a platelet count between 50 and 150×10^9/L and is generally asymptomatic. Moderate thrombocytopenia (platelet count between 20 and 50×10^9/L) is associated with an increased bleeding tendency in surgery; severe thrombocytopenia (platelet count $<20 \times 10^9$/L) can even cause severe spontaneous bleeding. While thrombocytopenia occasionally occurs with conventional chemotherapy for solid tumors, it becomes a major clinical problem when dose-intensive myelosuppressive therapies are used [1]. Additionally, thrombocytopenia is a defining characteristic of idiopathic thrombocytopenia purpura (ITP) and a major problem associated with myelodysplastic syndrome (MDS), acquired immunodeficiency syndrome (AIDS) and chronic liver disease [2–5]. The chronic thrombocytopenia observed in these patients is caused by defective or diminished platelet production or enhanced immunologic or non-immunologic platelet destruction and may be associated with abnormal platelet function [2–5].

The main treatment for thrombocytopenia owing to decreased or defective platelet production is platelet transfusion, although this remains an expensive and time-consuming strategy that has limited efficacy (allo-immunization) and is still associated with several risks. In the United States, platelet utilization doubled from 4 million units transfused in 1982 to more than 8 million units transfused in 1992 [6]. This trend continued in the 1990s, as the number of platelet units transfused increased by 40% annually [7]. Thus, the search for novel thrombopoietic growth factors, to improve primary hemostasis and, eventually, reduce the need for platelet transfusion is still warranted. A broad spectrum of novel thrombopoietic agents are currently being tested; with most of them working via the cytokine thrombopoietin (THPO) pathway, however, other molecules have been identified that influence megakaryopoiesis by a different, THPO-independent manner. This review will discuss the current status of new drugs for the treatment of thrombocytopenia, based upon different THPO receptor

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agonists and the preclinical development and therapeutic potential of the recently discovered regulators of megakaryopoiesis.

Megakaryopoiesis

Megakaryocytes (MKs) evolve from hematopoietic stem cells (HSCs) to become the largest cells of the bone marrow (BM). During this process, the megakaryocyte undergoes significant and dramatic changes, in order, ultimately, to form its progeny, platelets (Figure 1). Three sequential MK maturation stages have been identified by ultrastructural examination [8]. MK stage I (megakaryoblast) is the first morphologically recognizable MK cell that still resembles the pluripotent HSC, with a high nucleus:cytoplasm ratio, immature chromatin, prominent nucleoli and a small rim of basophilic cytoplasm. At the next maturation stage, MK stage II, the nucleus displays a budding shape, having nearly completed its final ploidy maturation, and the cytoplasm extends. Finally, MK stage III is the platelet-forming stage: the cell reaches its maximum size and the nucleus is polylobulated. Platelet production by MKs is a poorly understood event. Substantial experimental evidence supports a model, initially proposed in the 1970s and 1980s [9], wherein differentiated MKs extrude long cytoplasmic processes ('proplatelets') that serve as the immediate precursors of circulating platelets [10]. Recently, Junt *et al.* [11] reported a dynamic visualization technique, based upon multiphoton intravital microscopy that is able to study thrombopoiesis within the BM *in vivo*. By using this technique, they could confirm the concept of proplatelet formation *in vivo*.

The key physiological regulator of thrombopoiesis is THPO. THPO is synthesized primarily in the liver as a single 353-amino acid precursor protein and is secreted into the circulation without needing to be in a storage form [12]. On removal of the 21-amino acid signal peptide, the mature molecule consists of two domains: a receptor-binding domain that shows considerable homology to erythropoietin (EPO) and a carbohydrate-rich carboxy-terminus that is highly glycosylated and important in maintaining protein stability [13]. The gene of THPO was cloned in 1994 and is located on chromosome 3q27 [14]. Its receptor MPL is detected specifically on primitive hematopoietic stem cells [15] and on MKs and platelets [16]. Knockout mice lacking either MPL or its ligand, THPO, develop severe thrombocytopenia, but platelet levels are maintained at approximately 10–15% of normal values [17]. This profound thrombocytopenia is owing to a greatly reduced number of megakaryocyte progenitors and mature MKs, as well as a reduced polyploidy of the remaining MKs. A similar phenotype occurs in patients with congenital amegakaryocytic thrombocytopenia (CAMT) who have nonsense or missense mutations that severely reduce, or obliterate, the activity of MPL [18]. Plasma concentrations of THPO normally vary inversely with platelet count [19], rising to maximal levels within 24 h of the onset of profound thrombocytopenia, whereas in ITP, THPO levels are low despite low platelet counts [20]. THPO production is constitutive, but its removal, and, hence, the level remaining in the blood to affect megakaryopoiesis, is determined by the mass of MPL receptors present on platelets and MKs accessible in the plasma [21]. Another model suggests that THPO expression also seems to be regulated by the platelet count, at least in the marrow; very low platelet counts can upregulate THPO-specific mRNA expression [22]. The mechanism for this is still under investigation. THPO not only promotes the maturation of MKs, it also supports the survival and expansion of HSCs and all types of progenitor cells that display MK potential. THPO acts synergistally with the early acting growth factors, Flt3 ligand, c-kit ligand and interleukin-3 (IL-3), to stimulate the proliferation of primitive HSCs directly [23,24]. Despite its activity on HSCs and early megakaryopoiesis, THPO has little effect on platelet function and on the late stages of megakaryocyte development and platelet formation [25,26].

Besides THPO, many other cytokines have been postulated to participate in regulating megakaryopoiesis, including IL-3, stem cell factor (SCF), IL-6, IL-11 and many others [27–29]. Stromal cell-derived factor 1 (SDF1) and its Gi protein-coupled chemokine receptor, CXCR4, stimulate the homing of megakaryocytic progenitors [30]. In the 1990s, significant advances were made in

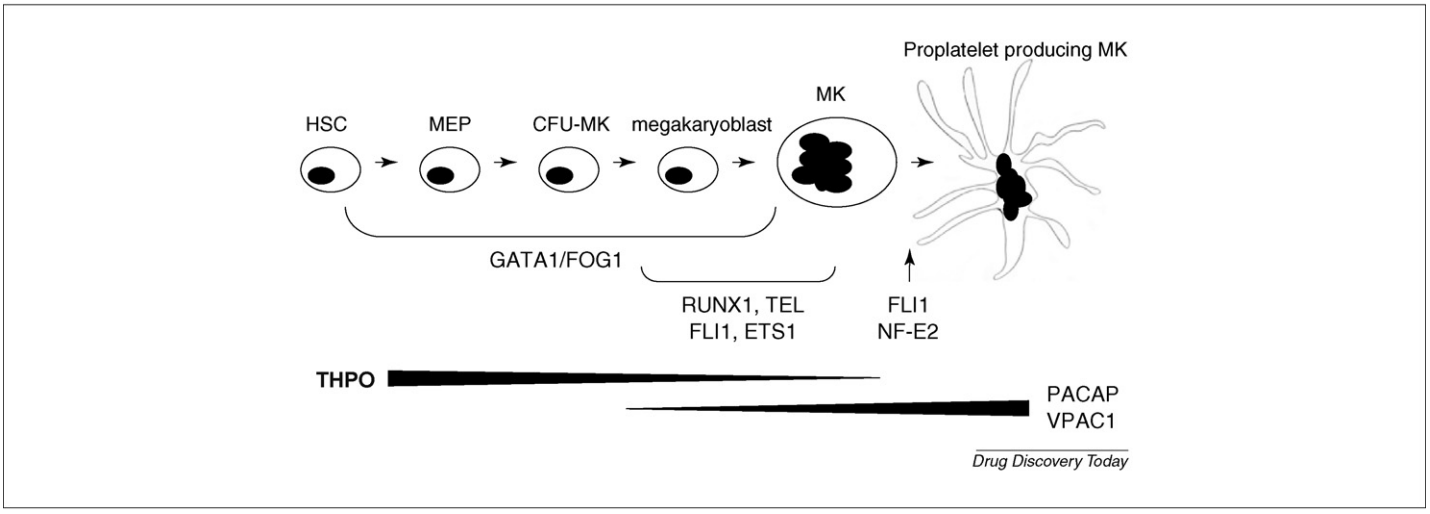

FIGURE 1

Overview of megakaryopoiesis. MKs evolve from HSCs in the BM and undergo dramatic changes to form platelets. HSC, hematopoietic stem cell; MEP, megakaryocyte erythrocyte progenitor; CFU-MK, colony forming unit megakaryocyte. Stages in which the transcription factors GATA1, FOG1, RUNX1, TEL, ETS1, FLI1 and NF-E2 are involved, are indicated. THPO's effect on megakaryopoiesis is mainly situated in the earlier stages whereas PACAP/VPAC1 signaling is mainly situated in the later stages.

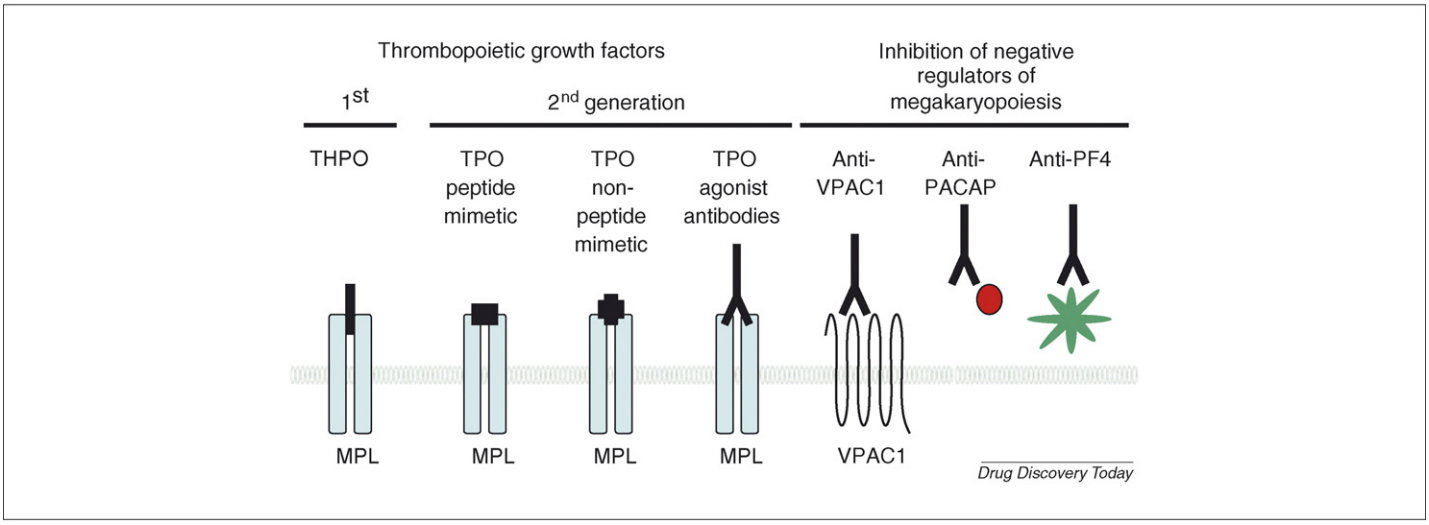

FIGURE 2

Current drug development of thrombocytopenia. Development of the first generation THPO receptor agonists was discontinued. The second generation agents contain THPO peptide and non-peptide mimetics as well as THPO receptor agonist antibodies. Inhibition of negative regulators of megakaryopoiesis by means of antibodies are the most recent and are still only in pre-clinical development.

understanding the transcriptional basis of hematopoietic cell differentiation [31]. The particular importance of erythro-MK transcriptional regulators, for example GATA1 and FOG1, in certain stages of MK and platelet differentation, has been described. The degree of thrombocytopenia induced in the different mouse knockouts for these transcriptional regulators seems to be more severe than for either MPL or THPO knockout mice. The X-linked transcription factor GATA1 is the founding member of the GATA-binding family of transcription factors and has been shown to play an essential role in normal erythropoiesis and megakaryocyte differentiation [32]. Vyas *et al.* [33] characterized the macrothrombocytopenia in GATA1 knockout mice with abnormal platelet numbers and ultrastructure and moderate defects in platelet activation. In contrast to GATA1 deficiency with late megakaryocyte differentiation problems, loss of FOG1 (Friend of GATA1) leads to specific ablation of the megakaryocytic lineage [34]. Other transcription factors which play a role during megakaryopoiesis include FLI1, NF-E2, ETS1, TEL and RUNX1 and are reviewed in [35].

First generation thrombopoietic growth factors

The first generation THPO receptor agonists consisted of recombinant THPO (rhTHPO) and 'megakaryocyte growth and development factor', the truncated and PEGylated (PEG; polyethylene glycol) THPO molecule (PEG-rHuMGDF). rhTHPO was produced in mammalian CHO cells, has the same amino acid sequence as endogenous THPO and is highly glycosylated. Nonetheless, the molecular weight of rhTHPO (90kDa) is 5 kDa less than endogenous THPO (95 kDa) [36]. PEG-rHuMGDF was produced in *Escherichia coli* and consisted of the receptor-binding, 163 amino-terminal amino acids of native THPO. It is conjugated to a 20 kDa PEG moiety to increase its circulatory half-life and possesses all the biologic activity of native THPO. Both products have undergone considerable preclinical and clinical evaluation (reviewed in [37]). The development of both products was discontinued in 1998 owing to the formation of neutralizing antibodies against endogenous THPO after PEG-rHuMGDF administration causing thrombocytopenia in healthy volunteers [36] and abrogation of its pharmacologic effect in cancer patients [38].

Second generation thrombopoietic growth factors

Because of the neutralizing antibodies that were detected following the administration of PEG-rHuMGDF, several THPO mimetics were developed that were non-immunogenic (Figure 2). One of the first group of second generation THPO mimetics are peptides that contain the THPO receptor-activating peptide designed in the complementary defining regions of a fragment antigen binding (Fab) (Fab 59), in an IgG Fc (AMG 531) or PEG (GW395058 and RWJ-800088) sequence. A second group consist of oral, non-peptide mimetics, which activate the THPO receptor by a different mechanism from THPO and, thus, have an additional effect; these mimetics include eltrombopag, AKR-501, LGD-4665, SB-559448, JTZ-132 and NIP-004. The third and final group of second generation THPO mimetics consists of THPO agonist antibodies which activate MPL but are modified in size (THPO minibodies, for example VB22B sc(Fv)2, or Ig type, e.g. MA01G4344). All second generation THPO mimetics stimulate THPO dependent cell lines via JAK-STAT signaling and increase platelet counts in animals. An overview of the discussed drugs is given in Table 1.

THPO peptide mimetics

Peptide libraries were screened for sequences that seem to stimulate THPO-dependent cell lines without any homology with endogenous THPO. Such sequences as the 14-amino acid peptide, Ile-Glu-Gly-Pro-Thr-Leu-Arg-Gln-Trp-Leu-Ala-Ala-Arg-Ala, were identified [39] but were too short-lived in the circulation to have any therapeutic effect. To prolong their biological activity, peptides were inserted into human Fab or Fc constructs or were pegylated. The sequence of this 14-amino acid peptide was modified at two residues, a pegylated dimer of this peptide was subsequently generated. Studies with this compound, GW395058, were performed to exclude the immunogenicity and cross-reaction of such peptides with human THPO [40].

TABLE 1

Overview of the discussed thrombopoietic drugs

1st generation THPO receptor agonists
rhTHPO
PEG-rHuMGDF
2nd generation THPO receptor agonists
Peptide
GW395058
Fab 59
AMG 531
RWJ-800088
Non-peptide
eltrombopag
AKR-501
LGD-4665
SB-559448
JTZ-132
NIP-004
Agonist antibodies
VB22B sc(Fv)2
MA01G4344
Inhibition of negative regulators of megakaryopoiesis
anti-PACAP
anti-VPAC1
anti-PF4

Fab 59 was constructed by engrafting 2 of the above described 14-amino acid THPO receptor agonist peptides into the complementarity-determining regions of a human Fab [41]. Fab 59 did stimulate the growth of THPO-dependent cell lines in a similar way to rhTHPO, but upon injection into mice, was not able to elevate the platelet count to the same high level as rhTHPO. Studies in humans have not yet been reported.

AMG 531 (romiplostim – Nplate™; Amgen) is a 'peptibody' of 60 kDa, composed of two disulphide-bonded human IgG1 κ-heavy chain constant regions (a Fc fragment) with two identical peptide sequences linked covalently at residue 228 of the heavy chain with the use of polyglycine (Figure 3) [42]. The Fc part extends the life of the molecule in the circulation to a half-life of 20 h. AMG 531 competes with THPO for MPL binding and signals via JAK-STAT; p38 MAPK and AKT pathways, as does THPO itself [43]. Preclinical studies have shown the ability of AMG 531 to increase platelet counts in wild-type mice [43] and to reduce the duration and severity of thrombocytopenia in murine models of chemotherapy- and radiation-induced thrombocytopenia [44]. AMG 531 was the first of the second generation thrombopoietic agents evaluated in clinical trials. Based on pharmacokinetic modeling from the rhesus monkey studies, phase I studies in healthy humans started with a single dose of 10 μg/kg intravenously. Unexpectedly, this produced peak platelet counts greater than 1000×10^9/L [42]. This robust effect was probably owing to the much higher affinity of AMG 531 for human MPL than monkey MPL. Subsequent doses from 0.1 to 2.0 μg/kg demonstrated a dose-dependent rise in platelet count, starting at day 5 and peaking at days 12–15 [42]. A clinically effective dose of 1 μg/kg was identified that increased the platelet count twofold. No antibodies were developed against THPO and intravenous and subcutaneous administration routes gave identical responses. Subsequently, several studies have been performed with AMG 531, mainly in patients with ITP, splenectomized and nonsplenectomized [45–47]. AMG 531 was administered subcutaneously once-weekly to patients with chronic, relatively severe ITP (platelet count $<30 \times 10^9$/L) and response was defined as achieving a platelet count of 50×10^9/L and doubling of the initial count. In summary, patients tolerated AMG 531 well at the doses tested and no anti-AMG 531 or anti-THPO antibodies were detected. Doses equivalent to 1 μg/kg or more, increased platelet counts to efficacious levels in ITP patients, with the median time to a platelet increase ranging from 5 to 10 days [47]. At this moment, phase 1–3 trials for ITP treatment have been completed with success.

Another strategy that has been used to increase the half-life of the THPO receptor agonist, is pegylation [48]. RWJ-800088 (formerly Peg-TPOmp) is such a molecule that is active at picomolar concentrations in cell-based assays. The molecule is composed of two identical 14-amino acid chains of 3295 Da joined by a lysyl residue and linked at each N-terminal tot a 20 kDa PEG chain. This is also the amino acid sequence, which was previously used for the construction for Fab 59. A phase I study was conducted in healthy human volunteers and has only very recently been published [48]. RWJ-800088 was administered as a single intravenous injection. At doses ≥0.75 μg/kg, platelet levels showed dose-related elevation as compared to placebo, reaching peak levels at days 10–12 and returned to baseline within 3–4 weeks. The two highest doses of RWJ-800088 appeared to increase burst-forming unit erythroid and colony-forming unit counts, suggesting some effect on progenitor lineages. RWJ-800088 is the first THPO mimetic peptide to show pluripotent effects in humans. RWJ-800088 was well tolerated, with no evidence of antibody formation in this single-dose study. Antibody formation is not, however, likely after a single dose of a peptide and, therefore, further assessment will be required after repeated doses of RWJ-800088 to confirm this finding. Doses above 1.5 μg/kg resulted in a dose-dependent rise in endogenous THPO levels, with the maximum occurring on day 3, indicating displacement from platelets and thereby a reduction in the rate of THPO clearance. An increase in platelet levels in

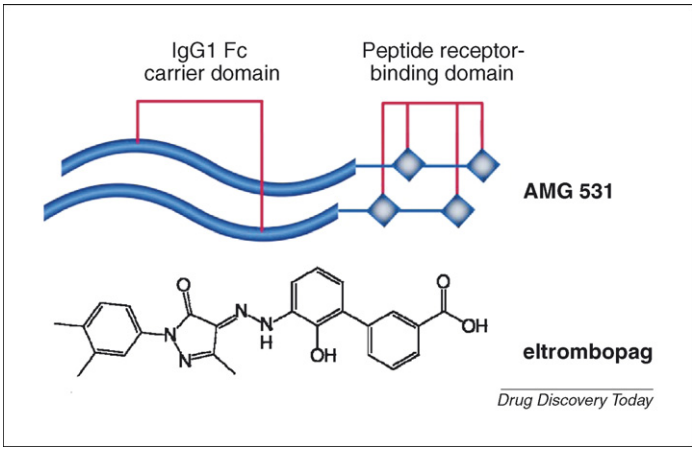

FIGURE 3

Structure of AMG 531 and eltrombopag. Both of these agents have been intensively studied in patients. AMG 531 is a 'peptibody' of 60 kDa composed of a Fc fragment with 4 identical peptide sequences. Eltrombopag (3′-{*N*′-[1-(3,4-dimethyl-phenyl)-3-methyl-5-oxo-1,5-dihydropyrazol-4-ylidene]hydrazino}-2′-hydroxybiphenyl-3-carboxylic acid) is a small hydrazone molecule (MW = 564 Da) which is orally deliverable. It consists of an acid group on one end, lipophilic groups on the other end and a metal chelate group in the centre.

combination with increased THPO-dependent platelet activation could potentially trigger platelet aggregation, resulting in thrombosis. Although THPO-dependent platelet activation has been described *in vitro*, thrombosis has not been confirmed in animal or clinical studies [37,49]. A multiple-dose experiment is necessary to further explore the potential of this molecule, as well as a study in patients with various causes of thrombocytopenia.

THPO non-peptide mimetics

A large number of THPO non-peptide mimetics has been identified so far by screening libraries of small non-peptide, orally deliverable molecules that stimulate STAT signaling in THPO-dependent cell lines. These THPO mimetics are highly species specific, activate the THPO receptor in a manner different from rhTHPO and have an additional effect to THPO. Most of them are still in preclinical development, but eltrombopag (Promacta® – Revolade™; GlaxoSmithKline) and AKR-501 (AkaRx Inc.) are currently being tested in humans. Because of their species restriction, there is limited information about toxicity and most efficacy studies have only been done in humans.

Eltrombopag (3′-{*N*′-[1-(3,4-dimethyl-phenyl)-3-methyl-5-oxo-1,5-dihydropyrazol-4-ylidene]hydrazino{-2′-hydroxybiphenyl-3-carboxylic acid) is a small hydrazone molecule (MW =564 Da). It consists of an acid group on one end, lipophilic groups on the other end and a metal chelating group in the centre. This structure is orally available and is given once daily (Figure 3). Initial studies showed that daily doses of 30, 50 and 75 mg were active in increasing the platelet count in healthy volunteers [50]. The results of the phase 1–3 trials of eltrombopag have been reported in patients with relapsed or refractory ITP [51]. In the groups receiving 30, 50 and 75 mg eltrombopag per day, the primary end point (platelet count of 50×10^9/L on day 43) was achieved in 28%, 70% and 81% of the patients, respectively. In the placebo group, the end point was achieved in 11% of the patients. A phase 2 study has recently been reported in patients with liver cirrhosis associated with Hepatitis C infection [52]. Patients with active hepatitis C commonly experience worsened thrombocytopenia from interferon therapy, compromising the ability to administer adequate doses. In this randomized, placebo-controlled clinical trial, eltrombopag increased platelet counts in a dose-dependent manner, thereby permitting the initiation of full-dose antiviral therapy with an enhanced remission rate and without creating an increased risk for bleeding complications [52]. Seventeen percent of patients with chronic liver disease from Hepatitis C infection have thrombocytopenia [53] and the prevalence of thrombocytopenia correlates with the severity of liver disease [54]. Studies with eltrombopag suggest a model wherein binding of THPO non-peptide mimetics to MPL either induces dimerization of MPL or directly activates the MPL downstream signal transduction cascade [55,56]. In contrast to peptide THPO mimetics, non-peptide THPO mimetics do not compete for binding with rhTHPO. Together, these findings suggested that many THPO non-peptide mimetics bind to MPL at a distance from the binding site for THPO and appear to initiate signal transduction by a mechanism different from rhTHPO. It is therefore not surprising that the effect of many of these THPO non-peptide mimetics is additive to the effect of rhTHPO. For example, in mice engrafted with human fetal liver $CD34^+$ cells, human platelet production stimulated by rhTHPO could be increased further by the addition of AKR-501, the next discussed THPO non-peptide mimetic [57].

AKR-501 (formerly YM477) is, together with AMG 531 and eltrombopag, one of the few THPO mimetics that has been evaluated in humans. Desjardins *et al.* [58] reported a rise in platelet count, in healthy volunteers, by as much as 1.75 times the baseline platelet count, even after only a single dose of 20 mg. Daily oral doses of 3, 10 or 20 mg for 14 days increased the platelet count by a factor 1.3, 2.25 and 2.8, respectively. These findings suggest that AKR-501 may be more effective than eltrombopag. Studies in ITP are still ongoing and clinical evaluation in patients with liver disease and chemotherapy are planned.

A phase II study of the small-molecule THPO mimetic, LGD-4665 (Ligand Pharmaceuticals Inc.) was recently initiated. The double-blind placebo-controlled trial is designed to evaluate the safety and efficacy of LGD-4665 in adult ITP patients over six weeks of treatment. Previously, they reported a single-center, randomized, placebo-controlled, double-blinded study in healthy male volunteers after single and multiple doses. Following single dose administration, a statistically significant platelet rise compared to placebo was observed following a 40 mg LGD-4665 dose. This THPO mimetic was well tolerated [59].

SB-559448 is an oral, non-peptide, small molecule THPO mimetic which also has been investigated in healthy volunteers. Su *et al.* reported a dose-dependent increase in platelet count with demonstrated safety [60].

Finally, two other non-peptide mimetics have been reported, but haven't entered in studies in humans yet, namely JTZ-132 [61] and NIP-004 [62]. JTZ-132 induced growth and differentiation of megakaryocytic progenitor cells and gave rise to higher platelet numbers at nadir and accelerated platelet recovery [61]. In mice receiving transplants of cord blood-derived $CD34^+$ cells, NIP-004 increased human megakaryoblasts, mature megakaryocytes and circulating human platelets sixfold [62]. NIP-004 is species specific.

THPO receptor agonist antibodies

Antibodies have been shown to be valuable therapeutics in the clinical treatment of various diseases, with a low level of serious adverse effects as a result of their intrinsic features, such as specific binding to the target antigen with high affinity, a long half-life and clinical safety as serum proteins. Owing to the size of the immunoglobulin molecule, agonist antibodies against cell receptors may interfere with receptor binding and dimerization. Orita *et al.* [63] reported that IgG antibodies against MPL, with no, or very weak, agonist activity, can be engineered to be agonist minibodies, (including diabodies or sc(Fv)2) as potent as the natural ligand. The non-covalent 'diabody' which has a comparable size to a Fab fragment, was generated by taking the VH and VL of a whole anti-MPL monoclonal IgG (VB22B) and coupling them via a 5-amino acid linker. This smaller molecule had a markedly increased agonist activity compared with its original full-length antibody. The corresponding VB22B sc(Fv)2 'minibody' was developed by binding one VH and one VL fragment together with a 15-amino acid linker and then binding 2 of these constructs together with the same 15-amino acid linker. VB22B sc(Fv)2 was injected into cynomolgus monkeys, had a half-life of 8–9 h and increased the platelet count. No antibodies were formed against this minibody, despite the species differences [63].

Recently, it has been reported that it may not just be the size, but also the structure of the immunoglobin agonist that is important for activating the MPL receptor. Kai *et al.* [64] highlight the importance of the hinge region in modulating THPO receptor agonist activity. They made MA01, an IgG1 anti-MPL monoclonal antibody that was only capable of weakly stimulating the growth of a THPO-dependent cell line. To reduce antibody-dependent cellular toxicity and complement-dependent cytotoxicity, the constant heavy chain (CH) region of MA01 was converted to IgG4 (MA01G4), which did not affect binding affinity or agonist activity. Agonist activity increased 10-fold when the hinge region was converted from IgG1 to IgG3 (MA01G4344). The IgG3 hinge is longer and more flexible than those of the other subclasses and this flexibility might promote receptor dimerization [65]. The hinge conversion probably modulates the physical relationship between the two antigen binding domains. MA01G4344 stimulated a THPO-dependent cell line and increased MK colony-forming cells (MK-CFCs) *in vitro*. A single injection increased the platelet count in human MPL transgenic mice for longer than 1 month. Studies in humans with THPO receptor agonist antibodies have not yet been reported.

Other regulators of thrombopoiesis to treat thrombocytopenia

IL-11 is a cytokine with pleiotropic effects on multiple tissues. It was first characterized as a hematopoietic cytokine with thrombopoietic activity, causing proliferation of megakaryocytic progenitor cells [66–68] and inducing megakaryocytic maturation [69] but has later been shown to be expressed in multiple other tissues, including brain, spinal cord neurons, gut and testis [70]. IL-11 is a 19 kDa protein and acts synergistically with IL-3, THPO and SCF to increase the number and maturation of megakaryocytic progenitors [71]. IL-11 induces maturation of early MKs by increasing MK size and ploidy [68]. This effect is dose related and is characterized by increases in peripheral platelet counts, which peak approximately 14–21 days after commencement [72]. Recombinant human interleukin-11 (rhIL-11, Oprelvekin, Neumega®; Wyeth) has been approved in the US by the Food and Drug Administration (FDA) to prevent severe thrombocytopenia and to reduce the need for platelet transfusion following myelosuppressive chemotherapy for non-myeloid malignancies [73–75]. In these patients, rhIL-11 has proven to be fairly effective, but with significant side effects. Preliminary studies have also shown that the use of rhIL-11 can be safely extended tot include both myeloid malignancies as well as those with bone marrow failure states including severe aplastic anemia and myelodysplatic syndromes [76–78]. Additionally, studies have been performed to test the efficacy of rhIL-11 to increase platelet counts in chronic liver disease [79–81]. These studies show that rhIL-11 can improve platelet counts in patients with early cirrhosis and these patients could benefit from rhIL-11 treatment. Just like in previous studies, side effects were significant, such as fluid retention, whose management may become a problem especially in patients with decompensated cirrhosis. Because this product carries this risk of serious side effects such as fluid retention and anaphylaxis, the development of other treatments of thrombocytopenia is still required.

A new treatment for ITP is PRTX-100, a highly purified form of Staphylococcal protein A (Protalex Inc.), which binds directly to monocytes and a subset of B-cells. Whereas drugs like eltrombopag or AMG 531 increase platelet counts by stimulating platelet production in ITP patients, this protein of 47 kDa targets the platelet destruction just like conventional therapies such as corticosteroids and intravenous immunoglobulines (IVIg). Yatko *et al.* [82] reported the inhibition of *in vitro* phagocytosis of platelets by monocytes. Studies on the use of PRTX-100 for ITP treatment are still ongoing.

Novel regulators of thrombopoiesis and their therapeutic potential

Recently, new strategies were discovered to treat thrombocytopenia in a less obvious way, by neutralizing the physiological inhibitors of megakaryopoiesis, for example platelet factor 4 (PF4) [83] and the pituitary adenylate cyclase-activating polypeptide (PACAP) and its receptor, VPAC1 [84]. PF4 is a 7.8 kDa protein that is primarily produced in MKs and expressed in platelets as a tetramer, where it comprises a significant proportion of the content of α-granules (2.5% on a molar basis) [85]. PF4 appears to function by binding with high affinity to glycosaminoglycans on cell surfaces [86]. Lambert *et al.* [83] reported that PF4 is a negative autocrine *in vivo* regulator of megakaryopoiesis. By using PF4 knockout mice and transgenic mice that overexpress human PF4, they found an inverse relationship between platelet count and the platelet PF4 content. Platelet half-life, nor THPO plasma levels were affected. mPF4 knockout mice had a shorter period of 5-Fluorouracil (5-FU)-induced thrombocytopenia, this period was longer in the hPF4 overexpressing mice. The former effect could also be established using function-blocking anti-PF4 antibodies, namely F(ab')2 fragments, since the full length anti-murine and anti-human PF4 antibodies unexpectedly accelerated a fall in platelet counts during the first week after combined therapy with 5-FU. The mechanism by which PF4 is released during megakaryopoiesis requires further studies and may occur continuously and/or during platelet release. Additional studies are needed to identify definitively not only the target cell for PF4 action, but also the receptor involved in this process. Other animal models with thrombocytopenia can be used to explore further the therapeutic potential of these anti-PF4 antibodies.

Our group recently found the neuropeptide PACAP and its Gs-coupled receptor VPAC1 to be negative regulators of megakaryopoiesis [84]. Freson *et al.* previously found an important inhibitory role for PACAP and its receptor VPAC1 in platelet function [87]. The patients studied had trisomy 18p and hence, three copies of the *PACAP* gene and elevated PACAP concentrations in plasma. The patients suffered from mental retardation, had a bleeding tendency with thrombopathy and a mild thrombocytopenia. The opposite phenotype (i.e. enhanced platelet reactivity) was observed in mice treated with the neutralizing monoclonal PACAP antibody PP1A4, with a polyclonal VPAC1 antibody and with a specific PACAP inhibitory peptide PACAP6-38 [87]. Since these patients also had a moderate thrombocytopenia, the question was posed whether the VPAC1 signaling cascade modulates megakaryopoiesis and platelet production. Stimulation of VPAC1 signaling, by its agonist PACAP and vasoactive intestinal peptide (VIP), was able to inhibit *in vitro* megakaryopoiesis: fewer MKs and a reduced polyploidy were found after differentiation of normal cord blood-derived CD34+ cells. The monoclonal antibodies PP1A4 (anti-PACAP/VIP) and 23A11 (anti-VPAC1) inhibited VPAC1 signaling. These antibodies stimulated megakaryopoiesis

in CFU-MK cultures of BM-derived Sca1+ cells from normal mice. The findings that inhibition of VPAC1 signaling stimulates *in vivo* platelet production and also platelet function, in contrast to MPL receptor agonists are very promising. This strategy can be used to treat congenital thrombocytopenia (GATA1 deficiency) and thrombocytopenia following myelosuppressive and myeloablative therapy [84]. PP1A4 or 23A11 stimulated platelet counts in mice and rabbits after chemotherapy-induced thrombocytopenia (busulfan treatment) in a THPO-independent manner. Blocking of VPAC1 signaling during chemotherapy-induced thrombocytopenia also enhanced platelet reactivity [87], which may be of substantial importance for hemostasis when platelet counts are that low. In a severe model of myeloablative therapy, mice were irradiated at levels that would normally be lethal and their survival, following treatment with PP144 was determined. Mice treated with PP1A4 had an increased survival percentage compared with non-treated mice. The fact that inhibition of VPAC1 signaling can reduce thrombocytopenia and enhance platelet recovery after myeloablative therapy, is very remarkable. THPO receptor agonists have been proven to show no clinical benefit, either in terms of ameliorating the platelet nadir or significantly reducing the need for platelet transfusion in several studies with myeoloablative therapy [88,89]. Additional experiments using larger animals will have to substantiate the present initial observations found for this novel regulator of megakaryopoiesis. The same article reports, for the first time, the partial rescue of the thrombocytopenia in GATA1 deficient mice by inhibition of VPAC1 signaling since their platelet count increased from 50 to $150(\pm 40) \times 10^9$/L after subcutaneous injection of 23A11. BM-derived CD34+ cells from a patient with severe thrombocytopenia owing to the GATA1 mutation D218Y [90] were differentiated *in vitro* into MKs. These GATA1-D218Y deficient MKs showed a maturation defect with immature Mks (mostly 2N phase), while adding 23A11 increased the DNA ploidy of these MKs.

The antibodies against these novel regulators of megakaryopoiesis still need extensive testing in toxicology studies. Before use in humans in further clinical evaluation, the immunogenicity of the mouse antibodies also needs to be reduced, by humanization or generating minibodies, as above described.

Conclusion

Some of the new thrombopoietic factors have undergone extensive clinical evaluation and were shown to increase significantly the platelet count in patients with ITP and hepatitis C-induced thrombocytopenia. The two most commonly used agents currently, AMG 531 and eltrombopag, seem to have few adverse effects, although long-term treatment studies need further evaluation. These agents could also be used in increasing the yield from platelet apheresis donors. The first generation thrombopoietic agents were able to increase platelet reconstitution after non-myeloablative therapy, however, not after myeloablative therapy. Whether this will also be the case for the second generation THPO receptor agonists still needs to be evaluated. Although studies with the 1st generation thrombopoietic factors suggest the clinical areas in which drugs with an identical mechanism of action such as AMG 531 may be effective, it needs to be emphasized that such predictions may not be true for the THPO non-peptide mimetics. Indeed, these THPO non-peptide mimetics have a unique mechanism of activating the THPO receptor and their effect is additive to that of rhTHPO, they do not necessarily need to induce MPL dimerization and they do not compete with THPO for MPL. Thus, combination of peptide with non-peptide THPO mimetics might be an even more effective treatment for thrombocytopenia with more clinical areas in which the combination therapy can be useful. Also, endogenous THPO levels are usually high in settings of myeloablative therapy, meaning that the THPO non-peptide mimetics indeed might have an increased effect in these settings compared to THPO. Novel regulators of megakaryopoiesis have been recently discovered and they might be effective in treating thrombocytopenia after myeloablative therapy and in other settings where THPO mimetics could fail, such as congenital thrombocytopenia, for example GATA1 deficiency. Inhibition of VPAC1 signaling in mice already showed a faster platelet recovery after myeloablative therapy and increased the platelet count in GATA1-deficient mice. The development of other THPO non-peptide mimetics (e.g. AKR-501) and THPO receptor agonist antibodies is rapidly progressing and these molecules are currently being tested.

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