Expert Opinion

informa
healthcare

Eltrombopag: the discovery of a second generation thrombopoietin-receptor agonist

Roberto Stasi
Regina Apostolorum Hospital, Department of Medical Sciences, Via S Francesco, 50, 00041 Albano Laziale, Italy

Background: Eltrombopag (formerly SB497115) is a first-in-class, orally active thrombopoietin-receptor (TpoR) agonist that stimulates megakaryocyte proliferation and differentiation. This drug is now under investigation in several conditions characterized by thrombocytopenia. *Methods*: The scope of this review is to illustrate the results of the preclinical studies that led to the discovery and preclinical development of eltrombopag. Articles were identified by a computer-assisted search of the literature published in English. The bibliographies of all retrieved articles were hand searched for further relevant citations. *Results/conclusion*: The activity of eltrombopag is dependent on expression of the TpoR but does not compete with endogenous Tpo. *In vitro* experiments suggest that eltrombopag interacts with TpoR at a distance from the binding site for endogenous Tpo. Thrombopoietin receptor stimulation leads to activation of the Janus kinase 2 and signal transducer and activator of transcription (STAT) 5 pathways, ultimately stimulating proliferation and differentiation of primary human $CD34^+$ bone marrow cells into $CD41^+$ megakaryocytes and increased platelet production. Measurements in platelets in several species indicated that eltrombopag specifically activates only human and chimpanzee STAT pathways. These findings have provided the rationale for the use of eltrombopag in clinical trials.

Keywords: agonist, eltrombopag, megakaryocytes, platelets, receptor, thrombocytopenia, thrombopoietin

Expert Opin. Drug Discov. (2009) 4(1):85-93

1. Introduction

A key concept in treating thrombocytopenia is to eliminate the underlying problem and target the mechanisms of the thrombocytopenia, either an increased platelet destruction or an impaired megakaryopoiesis. The progress with cytokines in the management of thrombocytopenia owing to decreased platelet production has not yet paralleled that in the treatment of anemia and neutropenia. Although administration of recombinant human IL-11 has been approved to decrease the frequency of platelet transfusions following myelosuppressive chemotherapy, its primary physiological function is not regulation of megakaryopoiesis [1], and it is associated with undesirable side effects [2,3]. Therefore, there has long been a need to develop a more specific treatment that increases the platelet count. The first generation thrombopoietic agents, recombinant human thrombopoietin (Tpo) and pegylated recombinant human megakaryocyte growth and development factor (PEG-rHuMGDF, a truncated and nonglycosylated form of Tpo), were extensively tested for their ability to stimulate platelet production and overcome thrombocytopenia. Whereas these molecules were shown to significantly increase circulating platelet levels in mice, primates and humans [4], clinical trials were discontinued after the development of Tpo autoantibodies was observed in healthy volunteers receiving PEG-rHuMGDF [5], and did not yield a clinically approved therapeutic

10.1517/17460440802642484 © 2009 Informa UK Ltd ISSN 1746-0441

Tpo. Subsequent research has focused on the development of thrombopoietic agents that bear little structural similarity with native Tpo, and do not trigger autoimmune anti-Tpo antibodies. These second generation molecules include peptide Tpo-receptor (TpoR) agonists, nonpeptide TpoR agonists and TpoR agonist antibodies. A detailed description of the various classes of the new thrombopoietic growth factors has been given in recent reviews [6,7]. In this article we shall focus on the discovery and preclinical development of eltrombopag (formerly SB497115), a first-in-class, oral nonpeptide TpoR agonist. Eltrombopag is now in advanced clinical development for thrombocytopenia of various etiologies, and may soon be used in clinical practice.

2. The thrombopoietin receptor

Thrombopoietin, which is produced primarily in the liver, binds to a specific receptor. The TpoR gene, named *c-mpl*, was cloned in 1992 [8]. It is located on human chromosome 1p34 and on murine chromosome 4 [8-11]. In both human and mouse, the *c-mpl* gene contains 12 exons distributed over 15 kb. Its organization conforms closely to the pattern observed for the genes of other hematopoietic receptor family members [11-13]. The promoter region lacks conventional TATA and CAAT motifs, but contains consensus binding sequences for several transcriptional regulators including GATA and Ets factors that are implicated in the transcription control of megakaryocyte-specific genes. Site-directed mutagenesis experiments identify one GATA-1 and two Ets motifs that play a crucial role in *c-mpl* expression. Transactivation assays demonstrate that GATA-1, Ets-1 and Fli-1 efficiently transactivate the *c-mpl* promoter in heterologous cells [14].

The TpoR molecule is a member of the class I hematopoietic growth factor receptor superfamily, a group of integral membrane proteins characterized by two extracellular cytokine receptor homology (CHR) domains, a five amino acid sequence (WSXWS motif) close to the transmembrane domains and a lack of intrinsic kinase activity of the cytoplasmic regions [12]. These receptors are non-covalently associated with members of the Janus kinase (JAK) family of cytosolic tyrosine kinases.

Three isoforms of TpoR have been identified by cDNA sequencing (c-mpl-P, c-mpl-K and c-mpl-S), and they differ in their intracellular domains as a result of alternative splicing [8,15]. Of these, the P isoform is the only one demonstrated to be a functional receptor. After cleavage of the signal peptide, the mature wild type TpoR (P isoform) is composed of an extracellular domain of 466 amino acids, a transmembrane domain of 22 amino acids and intracellular domain of 122 amino acids. An extra inactive splice variant, mpl-del, lacks 24 amino acids in the extracellular domain (exon 9) [16].

Human TpoR (now also known as CD110) is glycosylated and has an apparent molecular mass of 85 – 92 kDa. It is expressed on platelets and megakaryocytes and, at a lesser density, on most other hematopoietic precursor cells [17]. Studies with human platelets indicate the presence of ~ 56 ± 17 receptors per platelet with an affinity of 163 ± 31 pmol/l [18]. Apart from bone marrow, spleen and fetal liver, nonhemopoietic tissues such as placenta and brain may express TpoR transcripts [19]. Their functional role in these latter organs is not fully clear.

Biochemical and crystallographic data show that Tpo binds only the distal cytokine receptor homology domains and thereby initiates signal transduction (**Figure 1**) [20,21]. These distal domains probably have an inhibitory role, as in their absence the TpoR is active [22]. Sophisticated *in vitro* studies have shown that the new nonpeptidyl Tpo receptor agonists have a different pattern of interaction with TpoR, binding the extracellular juxtamembrane and transmembrane domains of the receptor [23,24]. By analogy with the erythropoietin (Epo) receptor, Tpo-stimulated dimerization with cross-phosphorylation has been put forth as a model for transmembrane signaling by the TpoR [25]. Studies with the Epo receptor have demonstrated that the distal domains preform an inactive dimeric receptor in which the intracellular regions are sufficiently distant from each other to prevent phosphorylation and activation of JAK2 [26,27]. On Epo binding, the preformed dimeric receptor undergoes conformational changes to initiate signal transduction. The active JAK2 then phosphorylates tyrosine residues within the receptor itself, as well as downstream signal transducer and activator of transcription (STAT) 3, STAT5, phosphatidylinositol 3-kinase (PI3K), the Ras-mitogen-activated protein kinase (MAPK) pathway and extracellular signal regulated kinases-1 and -2. Other studies with the TpoR have shown that distinct factors of the signal transducers and activators of transcription family (STAT-1 – 3, and -5) and the cAMP-responsive element binding transcription factor are activated [28-30].

3. Discovery strategy and preclinical development of eltrombopag

3.1 Molecular structure and characterization

Eltrombopag is a member of the biarylhydrazone class of compounds, with an empirical formula of $C_{25}H_{22}N_4O_4$ and a molecular weight of ~ 442 daltons (**Figure 2**). The compound has 1 acidic end, 1 lipophilic end and a metal chelate group in the center [31]. In clinical studies it has been used as eltrombopag olamine, the bis-monoethanolamine salt form of eltrombopag.

Eltrombopag was first identified from high-throughput screening of small-molecule compound collections [32]. The functional activity of eltrombopag was based on assays using luciferase as a reporter gene. In such assays luciferase gene transcription is activated by STATs in Tpo-dependent cell lines. The cell lines used for these experiments were BAF3/hTpoR, 32D-mpl and UT-7/Tpo [32]. BAF3/hTpoR derives from

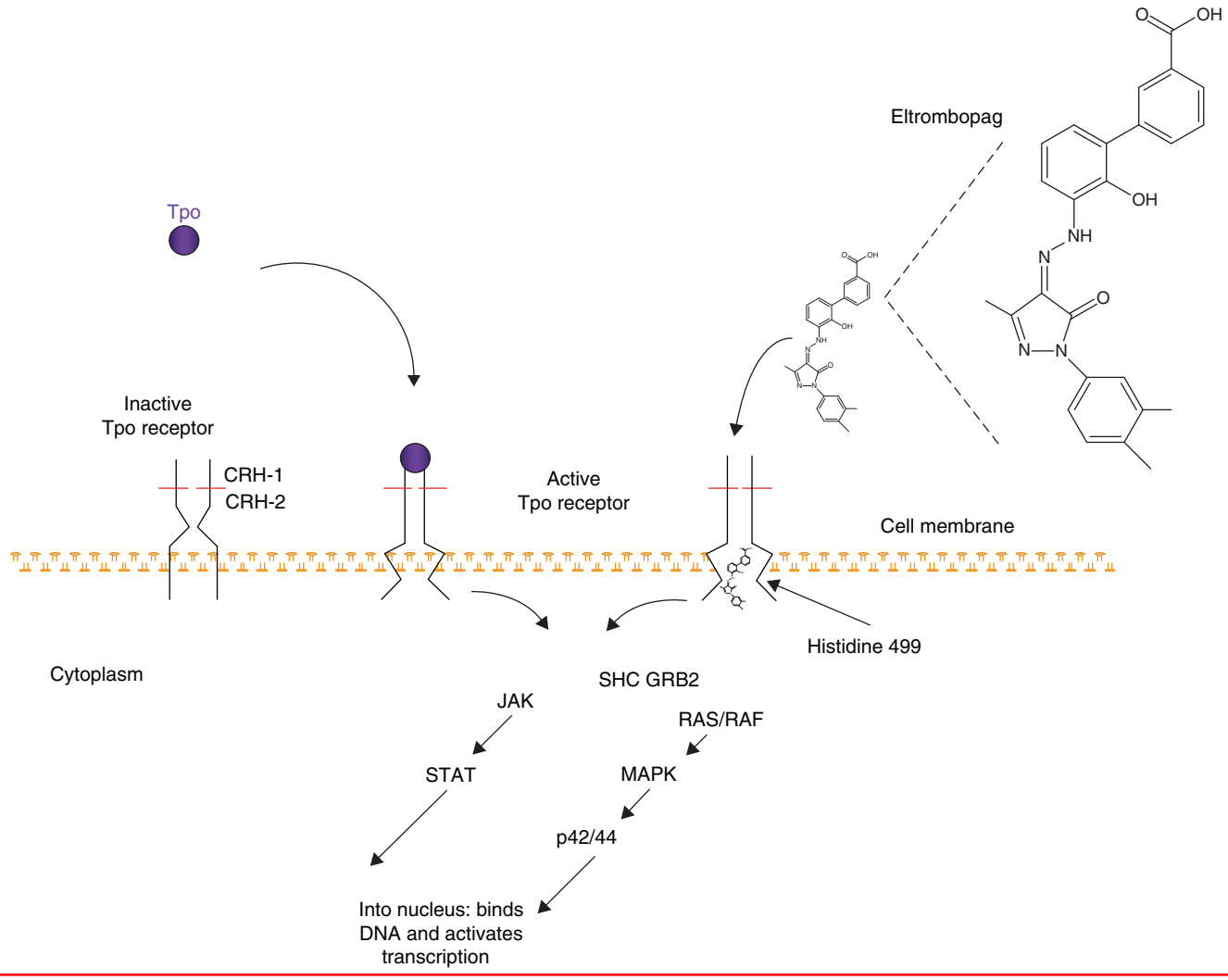

Figure 1. Mechanism of activation of the thrombopoietin receptor (TpoR). The TpoR has been proposed to exist as an inactive preformed dimer with a proximal (CRH-2) and distal (CRH-1) cytokine receptor homology domain. On binding of Tpo or romiplostim to the distal CRH-1 or binding of eltrombopag to the transmembrane region, the receptor conformation changes and several signal transduction pathways are activated that increase platelet production.

the IL-3-dependent mouse preB cell line BAF3 transfected with the Tpo-receptor and the STAT-inducible reporter interferon regulatory factor 1 (IRF-1) promoter coupled to a luciferase reporter gene and grown in recombinant mouse IL-3 (5 ng/ml) [32]. The 32D-mpl derives from an IL-3-dependent 32D (clone 3) mouse myeloid progenitor cell line transfected with the TpoR and the megakaryocyte-specific promoter gpIIb coupled to the luciferase reporter gene. UT-7/Tpo is a subline of UT-7, a cell line established from the bone marrow of a patient with acute megakaryoblastic leukemia. The cells express GPIIb/IIIa (CD41a), GPIb (CD42b), MY7 (CD13), MY9 (CD33), glycophorin A and PPO (platelet peroxidase activity). Morphologically UT-7/Tpo cells have the characteristics of mature megakaryocytes and the cells show high expression of PF4 and glycoprotein IIb. UT-7/Tpo cells can be maintained by Tpo alone. In the absence of Tpo, the majority of the cells die within a few days.

Eltrombopag demonstrated a half maximal effective concentration (EC_{50}) of 0.27 µM in murine BAF3/hTpoR cells transfected with the reporter gene luciferase. Eltrombopag and rhTpo both demonstrated activity in hTpoR-transfected 32D-mpl cells using the reporter assay with luciferase under the direction of the megakaryocyte-specific promoter gpIIb.

3.2 Specificity of eltrombopag binding

To determine whether the effect of eltrombopag was specific for the activation of the TpoR, cell lines that are dependent on other cytokines for growth were tested. In a variety of different assays, including electrophoretic mobility shift assay, eltrombopag showed no activity in cells expressing receptors for hematopoietic growth factors

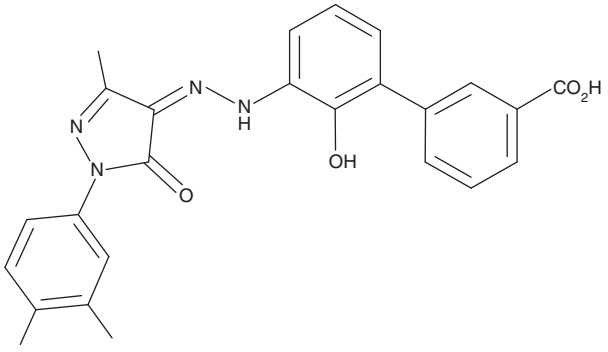

Figure 2. Structural formula of eltrombopag. The compound has an acidic (COOH) group at one end, lipophilic (CH3) groups at the other end and a metal chelate group in the center.

that are activated by STAT, including Epo, G-CSF, IFN-α, IFN-γ and IL-3 [32].

Dose-dependent STAT activation was detected 20 min after treatment of normal human platelets with 0.3 – 30 µm eltrombopag. However, there was no evidence of STAT activation in murine platelets following eltrombopag treatment. In fact, using platelets from several animal species, it was determined that the activity of eltrombopag was limited to human and chimpanzee platelets [32].

To explain the mechanism by which this species-specificity occurs, a series of cynomolgus monkey and human TpoR chimeric receptors were constructed in which the CRH1, CRH2 and the transmembrane and cytoplasmic domains were interchanged [23]. The functionality of all the chimeric receptors was confirmed by response to rhTpo. SKF-57626, a tool compound in the biarylhydrazone class, activated cells carrying the intact human TpoR but not the intact cynomolgus TpoR. When the monkey transmembrane domain replaced the human transmembrane domain, the human TpoR could no longer be activated; inserting the human transmembrane domain into the monkey TpoR allowed it now to be activated by SKF-57626. This was due mostly to a single amino acid difference in the transmembrane domain of the TpoR. Human and chimpanzee TpoR have a histidine at residue 499, whereas all the other species have a leucine. Changing this histidine to a leucine in the human TpoR causes loss of activity; changing the cynomolgus monkey TpoR leucine to a histidine makes the receptor now responsive to the mimetic. These results suggest that eltrombopag interacts with TpoR at a distance from the binding site for Tpo and seems to initiate signal transduction by a mechanism different from endogenous Tpo. Histidine 499 (and probably also threonine 496) in the transmembrane region is important either as a mediator of this effect or as the actual binding site of the mimetic. Furthermore, there does not seem to be any competition for binding with rhTpo.

Further indirect evidence to support this model derives from experiments involving mutations in the murine G-CSF receptor (mGCSFR), which has little homology with the human TpoR and does not bind significantly either compounds in the diazo class or rhTpo. An mGCSFR point mutation was tested that contained a His residue nine amino acids into the hydrophobic TM domain of GCSFR, corresponding to His499 in the human and chimpanzee TpoR. HepG2 cells transiently transfected with this receptor and the reporter gene construct responded to compound. A double mutation in which an extra residual three amino acids N-terminal to the His was replaced with Thr, as in the hTpoR, showed an increase in activity over the single mutation [23]. These results suggest that TpoR agonist compounds such as eltrombopag interact with His499, in addition to Thr496, to either change conformation of TpoR or induce dimerization, resulting in activation of the signal transduction pathways of TpoR and imparting biologically relevant function.

Other studies of the mechanism of action of eltrombopag and compounds bearing a similar pharmacophore suggest that association with metal ions (i.e., Zn^{2+}) is necessary for eltrombopag to activate the receptor [24,33].

3.3 Activation of thrombopoietin receptor signaling pathways

The gel electrophoresis mobility shift assay (EMSA) is used to detect protein complexes with nucleic acids. The EMSA technique is based on the observation that protein:DNA complexes migrate more slowly (are 'shifted') than free DNA molecules when subjected to non-denaturing polyacrylamide or agarose gel electrophoresis. Electrophoresis mobility shift assays were done on the BAF3/TpoR screening cell line, as well as on UT7-Tpo cell line to confirm that the activity induced by eltrombopag was acting through the JAK/STAT signal transduction pathway. As little as 0.01 µM eltrombopag activated STAT5; higher concentrations of compound shifted the IRF-1 probe in the assay in a dose-dependent manner [32]. The compound activated STATs with similar kinetics to Tpo; peak activity was demonstrated at 30 min and decreased to background levels at ~ 60 – 120 min. The p42/44 (erk1/2) and p38 MAPKs, previously reported to be activated by Tpo [34], as well as phosphorylation of p42/44 and p38 were demonstrated in lysates of BAF3/TpoR cells following 30 min of treatment with rhTpo or 30 µM eltrombopag. In addition to activation of the STAT and MAPK signal transduction paths, expression of the early response genes Fos, EGR-1 and *CIS* was also demonstrable with eltrombopag.

3.4 Stimulation of proliferation and differentiation in mammalian cells and cell lines

The cellular functions of proliferation and differentiation under eltrombopag exposure were examined. Proliferation assays were performed by measuring 5-bromo-2′-deoxyuridine (BrdU) incorporation in BAF3/TpoR cells and by the colorimetric tetrazolium (MTT) assay in UT7-Tpo cell lines. Eltrombopag had an EC_{50} of 30 and 40 nM in BAF3/TpoR and UT7-Tpo proliferation assays, respectively.

In addition to a proliferative effect on cells of the megakaryocytic lineage, eltrombopag also induces differentiation of hematopoietic stem cells into committed megakaryocyte progenitor cells. Assessment of megakaryocyte maturation was determined by measuring the appearance of the megakaryocyte-specific marker glycoprotein CD41 on human $CD34^+$ cells purified by magnetic separation from human bone marrow samples. The data were calculated as a percentage of the maximal number of cells responding to the maximally efficacious dose of rhTpo (i.e., 100 ng/ml). Eltrombopag increased the differentiation of bone marrow $CD34^+$ cells in a dose-dependent manner with an average EC_{50} of 200 nM in multiple experiments. The efficacy of the compound relative to Tpo max averaged 120% at 3 µM in > 20 individual marrow experiments.

Thymidine incorporation assays in N2C-Tpo cells were used to determine the effect of treatment with eltrombopag in combination with rhTpo [35]. There seemed to be an additive effect when eltrombopag was added to suboptimal amounts of rhTpo. The additive effect of eltrombopag also occurred in the presence of rhTpo at a concentration (100 ng/ml) that caused a plateau in cell proliferation rates.

3.5 Pharmacokinetics and metabolism

Limited pharmacokinetic data are available in chimpanzees. In healthy human subjects the time to maximum concentration (T_{max}) of eltrombopag is 2 – 6 h, with a half-life ($t_{½}$) of 21 – 32 h. The pharmacokinetic profile of this agent is linear for doses ≤ 200 mg, with proportional increases in the area under the curve (AUC) in doses ranging from 50 to 200 mg [36]. In a Phase I clinical trial eltrombopag demonstrated dose-dependent and linear pharmacokinetics in 73 healthy adult male volunteers (mean age, 27.5 years; mean baseline platelet count, 239×10^9/l) [37]. Volunteers were administered eltrombopag 5, 10, 20, 30, 50 or 75 mg/day or placebo for 10 days. In the 75-mg/day cohort, the maximum concentration (C_{max}) was 7.3 µg/ml, the corresponding T_{max} was 2.5 – 5 h and the mean AUC was 79.0 µg·h/ml. The mean $t_{½}$ was > 12 h for all doses except the 5-mg/day dose, which demonstrated a $t_{½}$ of 9 h. With multiple daily dosing, an accumulation of 40 – 50% was noted with doses ≥ 20 mg/day.

Eltrombopag is metabolized predominantly in the liver. Oxidation and conjugation with cysteine, glutathione or glucuronic acid were identified as the primary *in vitro* metabolic pathways. Eltrombopag inhibited the organic anion transporting polypeptide 1B1 (OATP1B1) *in vitro*. Organic anion transporting polypeptide 1B1 is a polyspecific transporter that mediates uptake of a variety of drugs into human hepatocytes. In healthy adult subjects, eltrombopag increased exposures of rosuvastatin, an OATP1B1 substrate. Plasma rosuvastatin C_{max} increased 2.03-fold and $AUC_{(0\rightarrow\infty)}$ increased by 55%. A reduced dose of rosuvastatin should be considered when coadministered with eltrombopag and careful monitoring for side effects should be undertaken. On the other hand, no clinically significant interactions were detected when eltrombopag and cytochrome P450 substrates, inducers or inhibitors were coadministered.

Limited pharmacology data suggest that in subjects of East-Asian descent (e.g., Japanese, Chinese, Korean etc.) exposure to eltrombopag, expressed as AUC, was ~ 70 – 80% higher compared to non-Asian individuals who were predominantly Caucasian [36]. Therefore, a dose reduction should be considered for this population.

4. Clinical development

Although clinical trials of eltrombopag are limited, they have convincingly demonstrated the efficacy of the drug in elevating platelet counts in patients with idiopathic thrombocytopenic purpura (ITP) and thrombocytopenia associated with hepatitis C virus (HCV) related cirrhosis.

The effects of eltrombopag in patients with chronic ITP (≥ 6-month history of ITP and documented platelet counts < 30×10^9/l at enrollment) have been assessed in a Phase II, placebo-controlled, double-blind trial (TRA100773A). Platelet counts were analyzed after 6 weeks of treatment with placebo or eltrombopag at doses of 30, 50 or 75 mg/day p.o. [38]. The results showed a dose-dependent increase in the proportion of responders with a statistically significant effect in the 50- and 75-mg arms compared with the placebo arm.

The preliminary results of other trials in chronic ITP have been reported only in abstract form. A randomized, double-blind, placebo-controlled Phase III trial (TRA100773B) enrolled 114 adults with chronic ITP and baseline platelet counts of < 30×10^9/l. These patients were randomized to either placebo (38 patients) or eltrombopag 50 mg/day (76 patients) for 6 weeks [39]. The eltrombopag dose could be increased to 75 mg in patients not responding after an initial 3 weeks of treatment. At the end of the trial, 16% of placebo patients and 59% of eltrombopag patients achieved the primary end point (platelet count ≥ 50×10^9/l).

Preliminary results of the continuing Extended Dosing Study (EXTEND) trial have been presented at the 2008 meeting of the European Hematology Association (EHA) [40]. The EXTEND study is an open-label extension study designed to assess the long-term safety and efficacy of eltrombopag. Patients previously enrolled in an eltrombopag study were eligible to enter this trial after a 4-week washout period. Eltrombopag was initiated at 50 mg/day and then the dose was adjusted according to the platelet count, with doses between 25 and 75 mg/day. Of 109 patients that started eltrombopag treatment, 80% achieved a platelet count of > 50×10^9/l at least once and 78% of these patients maintained platelets > 50×10^9/l for > 50% of their time in the study.

Further eltrombopag data presented at the 2008 EHA meeting showed that treatment with eltrombopag provided consistent and predictable platelet responses on repeated administration [41]. In the REPEAT (Repeat ExPosure to Eltrombopag in Adults with Idiopathic Thrombocytopenic Purpura) trial repeated intermittent use of eltrombopag

produced consistent and predictable responses in patients with chronic ITP, generating similar platelet counts with each subsequent cycle [41].

For RAISE (Randomized placebo-controlled ITP Study with Eltrombopag), a trial that has been recently closed to recruitment, results are not yet available. It is a global, randomized, double-blind, placebo-controlled Phase III trial that assessed the safety, efficacy and tolerability of eltrombopag in a long-term treatment setting (≤ 6 months) involving 189 patients across 135 centers in 26 countries.

The efficacy and safety of eltrombopag in HCV-positive cirrhotic patients with substantial thrombocytopenia (defined as a platelet count of 20 to < 70×10^9/l) has been assessed in a multicenter, prospective, randomized, controlled, Phase II trial [42]. Eligible patients were randomly allocated to receive eltrombopag (30, 50 or 75 mg/day) or placebo for a 4-week initial treatment phase. All patients who completed 4 weeks of initial treatment and had reached a platelet count of ≥ 70×10^9/l received antiviral therapy (pegylated interferon [PEG-IFN] with ribavirin) concomitant with eltrombopag or placebo for 8 weeks (extended to 12 weeks after preclinical safety data were obtained). The results suggest that eltrombopag, especially at a dose of 75 mg/day, is effective in raising platelet counts in cirrhotic HCV-positive patients, and it seems to be safe. Although platelet counts dropped after initiation of PEG-IFN therapy, despite continuation of eltrombopag, most patients were able to complete 12 weeks of antiviral treatment. A Phase III trial that will continue to define eltrombopag's efficacy and safety in this patient group has just begun.

Other studies are underway to evaluate the use of eltrombopag in patients with chemotherapy-induced thrombocytopenia.

5. Safety and tolerability

The principal nonclinical toxicology findings associated with eltrombopag administration include cataracts, renal toxicity and hepatotoxicity [36]. *In vitro* phototoxicity (3T3 and CHO cells) has also been observed. There was no evidence of genotoxicity *in vitro* in bacterial cells or in two *in vivo* assays. In mammalian cells *in vitro*, eltrombopag was weakly positive. The weight of evidence suggests that eltrombopag does not pose a genotoxic risk in humans. In addition, lifetime oral administration of eltrombopag was not associated with the induction of tumors in mice or rats. Eltrombopag was not teratogenic in rats or rabbits. In these species, however, eltrombopag does not bind the TpoR. There are no data about the teratogenicity of eltrombopag in humans and chimpanzees.

No serious adverse events were reported during both the clinical trials in patients with ITP and thrombocytopenia associated with HCV-related cirrhosis. The rate of adverse events did not differ among those who received the active drug and those who received placebo, and were not dose related. Headache has been consistently the most frequently reported adverse event in all eltrombopag trials. Because of the relatively small number of patients exposed to eltrombopag so far and the lack of long-term data, the manufacturer has proposed a RiskMAP system to address issues of long-term safety and efficacy [36]. If eltrombopag is approved, the RiskMAP program would include mandatory enrollment of prescribers and patients, controlled distribution, follow-up and monitoring and education for prescribers and patients.

6. Technology evaluation

6.1 Market overview

One recent review indicates an estimated incidence of chronic ITP in adults of 5.8 – 6.6/100,000/year in the US, with a similar incidence in the UK [43]. However, the population of patients with chronic ITP requiring active treatment and potentially targeted by eltrombopag is probably < 1/100,000/year [44]. In the US, the estimated prevalence of anti-HCV antibodies is 1.6% (95% CI, 1.3 – 1.9%), with a peak prevalence of 4.3% observed among persons 40 – 49 years of age [45]. Of these individuals, estimated at 3.4 million – 4.9 million people, ~ 55 – 85% have chronic infection that might need curative treatment [45]. Thrombo-cytopenia either preexists and prevents the initiation of treatment with PEG-IFN or develops as a consequence of PEG-IFN treatment, leading to dose modification in 19% of cases and discontinuation in 2% [46]. In patients with cirrhosis, thrombocytopenia complicates antiviral treatment much more frequently than in patients with HCV infection without cirrhosis [47].

6.2 How the technology works

High-throughput screening (HTS) is a technically sophisticated, yet random screening of proprietary in-house chemical libraries, typically smaller than 10^6 compounds, and accounts for the predominant part of all newly discovered small-molecule lead compounds [48]. It is quite demanding both in terms of resources (costs for robotic equipment and material consumption) and technical development (set up of sophisticated assays, storage and handling of the chemical archives). The compounds ('hits') resulting from *de novo* HTS screens typically have affinity/inhibition constants in the micromolar range, most likely owing to the fact that the hits do not optimally fit into the target protein's pocket, thus requiring time-consuming medicinal–chemical optimization steps to be developed to a final lead compound of appropriate affinity [49]. Computer-based 'virtual' screening was first considered to have the potential of at least partially replacing HTS. However, in most cases, this method has not enabled the *de novo* discovery of novel drug candidates, owing to intrinsic limitations in predicting binding energies in solution [50]. To overcome these limitations, fragment-based lead discovery approaches have gained importance in recent years, aiming at increasing the affinity of an initially low-affinity (typically millimolar) binder in a stepwise fashion either by increasing the molecular size of the initial hits or by combining two adjacently binding pharmacophores to form a new chemical entity [51]. The development of DNA-encoded chemical libraries,

combined with suitable selection and high-throughput sequencing strategies, holds promises to further fill this gap [52].

6.3 Alternative technologies

Phage display is a technology widely used in the development of new drugs. Large numbers of filamentous bacteriophages carrying random surface peptide sequences are produced by recombinant DNA techniques [53]. Immobilized receptor protein is used to pan for adherent phages. Using phages that express high levels of coat protein in a primary screen, a family of low-affinity binding peptides can be identified. Typically, combinatorial libraries of 10^9 phages can be conveniently screened. Once eluted from the immobilized receptor, the inserted peptide genes of such phage can be readily sequenced, yielding a consensus of the residues critical for binding. By next generating a library of sequences encoding these peptides, containing both the invariant amino acids derived from the first screen and a new group of variable amino acid sites, peptides of higher binding affinity can often be identified. After two or more such rounds of screening, individual peptides can be tested for cell-bound receptor binding and biological activity.

Another technology involves screening of peptide libraries for sequences that stimulate the growth of Tpo-dependent cell lines and that lack sequence homology with Tpo [54]. In fact, antibodies made against such mimetics would not cross-react with human Tpo [55]. These linear peptides were equipotent with Tpo in cell-based assays but were too short-lived in the circulation to have any therapeutic effect. This led to efforts to prolong their biologic activity by insertion into human Fab and Fc constructs. Romiplostim (formerly AMG 531) is a recombinant protein known as a peptibody. It is made up of two disulfide-bonded IgG1 heavy-chain and k light-chain constant regions Fc fragments, each of which is covalently bound at residue 228 of the heavy chain with two identical peptide sequences linked by means of polyglycine [56]. The carrier Fc component of the molecule binds to the neonatal Fc salvage receptor and undergoes endothelial recirculation, resulting in a substantially longer $t_{½}$ than that of the peptide only [57]. The peptide component binds to and activates the human TpoR.

There is no sequence homology of romiplostim to endogenous Tpo [56]. The reader is referred elsewhere for comprehensive reviews of the new molecules [6,7].

7. Conclusion

The ability of eltrombopag to activate the Tpo receptor have been demonstrated *in vitro* and in animal studies, and have provided the foundation for the clinical development of eltrombopag in a broad range of thrombocytopenia-associated conditions. Although available data suggest that eltrombopag initiates signal transduction by a mechanism different from endogenous Tpo, the final pathways of signal transduction seem to be the same for both molecules.

Collectively, the encouraging safety and efficacy data in Phase II and III studies involving patients with ITP and chronic liver disease have supported the further testing of eltrombopag in Phase III trials. Continuing maintenance studies will address the long-term efficacy and safety issues and will help define where this agent fits within the current treatment landscape.

8. Expert opinion

The new Tpo agents, including not only eltrombopag but also romiplostim and others in development, promise to revolutionize the management of many types of thrombocytopenia. In fact, stimulation of platelet production is a new approach to the treatment of patients with chronic ITP, which, traditionally, has aimed at decreasing platelet destruction. Both definitive and preliminary results of clinical trials investi-gating eltrombopag demonstrate the efficacy and favorable side effect profile of the drug in a high percentage of refractory patients with ITP. On the other hand, the management of thrombocytopenia in the setting of HCV infection usually involves dose reduction or interruption of PEG-IFN therapy rather than administration of a targeted therapy for thrombocytopenia, as is the case for neutropenia or anemia.

Although it has been recently been approved by the US FDA for use in patients with chronic ITP, so far eltrombopag has been given to patients for relatively short periods of time relative to the chronic and potentially lifelong course of the diseases associated with thrombocytopenia, which leaves concerns regarding its long-term use. It is also uncertain whether eltrombopag treatment can sustain platelet counts over years in chronic ITP, or over the entire duration of HCV therapy. This is being investigated in continuing clinical trials. Nevertheless, eltrombopag is potentially useful in those ITP patients needing a transient increase of the platelet count, such as in preparation of surgery.

Romiplostim, another thrombopoietic growth factor, has also been approved by FDA as well as by the European Agency for the Evaluation of Medicinal Products (EMEA) for use in patients with chronic ITP. Both eltrombopag and romiplostim seem to be very efficacious in increasing the platelet count of ITP patients. The presence of additive or synergistic effects, or of cross-resistance between the two agents, cannot be evaluated at this time, as exclusion criteria for trials involved previous treatment with another thrombopoiesis-stimulating agent. Also, the mechanisms of resistance to these drugs have not been clarified, and their investigation might unravel new clues to potential therapeutic targets.

Declaration of interest

RS received honoraria from GlaxoSmithKline and Amgen for participating in advisory boards.

Bibliography

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. Choi ES, Hokom MM, Chen JL, et al. The role of megakaryocyte growth and development factor in terminal stages of thrombopoiesis. Br J Haematol 1996;95:227-33
2. Gordon MS, Mccaskill-stevens WJ, Battiato LA, et al. A phase I trial of recombinant human interleukin-11 (neumega rhIL-11 growth factor) in women with breast cancer receiving chemotherapy. Blood 1996;87:3615-24
3. Tepler I, Elias L, Smith JW 2nd, et al. A randomized placebo-controlled trial of recombinant human interleukin-11 in cancer patients with severe thrombocytopenia due to chemotherapy. Blood 1996;87:3607-14
4. Kuter DJ, Begley CG. Recombinant human thrombopoietin: basic biology and evaluation of clinical studies. Blood 2002;100:3457-69
5. Li J, Yang C, Xia Y, et al. Thrombocytopenia caused by the development of antibodies to thrombopoietin. Blood 2001;98:3241-8
6. Kuter DJ. New thrombopoietic growth factors. Blood 2007;109:4607-16

•• **A comprehensive review about thrombopoietic agents.**

7. Evangelista ML, Amadori S, Stasi R. Biologic aspects of thrombopoietin and the development of novel thrombopoietic agents for clinical use. Current drug discovery technologies 2007;4:162-73

•• **An exhaustive review about the biology of thrompobopoietin and the development of thrombopoietic agents.**

8. Vigon I, Mornon JP, Cocault L, et al. Molecular cloning and characterization of MPL, the human homolog of the v-mpl oncogene: identification of a member of the hematopoietic growth factor receptor superfamily. Proc Natl Acad Sci USA 1992;89:5640-4
9. Skoda RC, Seldin DC, Chiang MK, et al. Murine c-mpl: a member of the hematopoietic growth factor receptor superfamily that transduces a proliferative signal. EMBO J 1993;12:2645-53
10. Le Coniat M, Souri M, Vigon I, et al. The human homolog of the myeloproliferative virus maps to chromosome band 1p34. Hum genet 1989;83:194-6
11. Alexander WS, Dunn AR. Structure and transcription of the genomic locus encoding murine c-Mpl, a receptor for thrombopoietin. Oncogene 1995;10:795-803
12. Bazan JF. Structural design and molecular evolution of a cytokine receptor superfamily. Proc Natl Acad Sci USA 1990;87:6934-8
13. Mignotte V, Vigon I, Boucher de Crevecoeur E, et al. Structure and transcription of the human c-mpl gene (MPL). Genomics 1994;20:5-12
14. Deveaux S, Filipe A, Lemarchandel V, et al. Analysis of the thrombopoietin receptor (MPL) promoter implicates GATA and Ets proteins in the coregulation of megakaryocyte-specific genes. Blood 1996;87:4678-85
15. Kiladjian JJ, Elkassar N, Hetet G, et al. Study of the thrombopoitin receptor in essential thrombocythemia. Leukemia 1997;11:1821-6
16. Li J, Sabath DF, Kuter DJ. Cloning and functional characterization of a novel c-mpl variant expressed in human CD34 cells and platelets. Cytokine 2000;12:835-44
17. Debili N, Wendling F, Cosman D, et al. The Mpl receptor is expressed in the megakaryocytic lineage from late progenitors to platelets. Blood 1995;85:391-401
18. Li J, Xia Y, Kuter DJ. Interaction of thrombopoietin with the platelet c-mpl receptor in plasma: binding, internalization, stability and pharmacokinetics. Br J Haematol 1999;106:345-56
19. Wolber EM, Jelkmann W. Thrombopoietin: the novel hepatic hormone. News Physiol Sci 2002;17:6-10
20. Alexander WS, Metcalf D, Dunn AR. Point mutations within a dimer interface homology domain of c-Mpl induce constitutive receptor activity and tumorigenicity. EMBO J 1995;14:5569-78
21. Feese MD, Tamada T, Kato Y, et al. Structure of the receptor-binding domain of human thrombopoietin determined by complexation with a neutralizing antibody fragment. Proc Natl Acad Sci USA 2004;101:1816-21
22. Sabath DF, Kaushansky K, Broudy VC. Deletion of the extracellular membrane-distal cytokine receptor homology module of Mpl results in constitutive cell growth and loss of thrombopoietin binding. Blood 1999;94:365-7
23. Erickson-Miller CL, Delorme E, Iskander M, et al. Species specificity and receptor domain interaction of a small molecule TPO receptor agonist [abstract 2909]. Blood 2004;104

•• **This study shows that eltrombopag binds the transmembrane domain of the TPO-receptor of humans and chimpanzees, but not the TPO-receptor of other species.**

24. Kim MJ, Park SH, Opella SJ, et al. NMR structural studies of interactions of a small, nonpeptidyl Tpo mimic with the thrombopoietin receptor extracellular juxtamembrane and transmembrane domains. J Biol Chem 2007;282:14253-61
25. Kaushansky K. The molecular mechanisms that control thrombopoiesis. J Clin Invest 2005;115:3339-47
26. Livnah O, Stura EA, Middleton SA, et al. Crystallographic evidence for preformed dimers of erythropoietin receptor before ligand activation. Science (NY) 1999;283:987-90
27. Remy I, Wilson IA, Michnick SW. Erythropoietin receptor activation by a ligand-induced conformation change. Science (NY) 1999;283:990-3
28. Tortolani PJ, Johnston JA, Bacon CM, et al. Thrombopoietin induces tyrosine phosphorylation and activation of the Janus kinase, JAK2. Blood 1995;85:3444-51
29. Kaushansky K. Thrombopoietin and the hematopoietic stem cell. Ann NY Acad Sci 2005;1044:139-41
30. Ezumi Y, Takayama H, Okuma M. Thrombopoietin, c-Mpl ligand, induces tyrosine phosphorylation of Tyk2, JAK2, and STAT3, and enhances agonists-induced aggregation in platelets in vitro. FEBS Lett 1995;374:48-52
31. Stasi R, Evangelista ML, Amadori S. Novel thrombopoietic agents: a review of their use in idiopathic thrombocytopenic purpura. Drugs 2008;68:901-12

• **A review of the pharmacology of romiplostim and eltrombopag and the use of the novel thrombopoietic agents in ITP.**

32. Erickson-Miller CL, Delorme E, Tian SS, et al. Discovery and characterization of a selective, nonpeptidyl thrombopoietin receptor agonist. Exp Hematol 2005;33:85-93

•• **A description of the laboratory investigations and preclinical strategies that lead to the development of eltrombopag.**

33. Duffy KJ, Price AT, Delorme E, et al. Identification of a pharmacophore for

thrombopoietic activity of small, non-peptidyl molecules. 2. Rational design of naphtho[1,2-d]imidazole thrombopoietin mimics. J Med Chem 2002;45:3576-8

34. Rojnuckarin P, Drachman JG, Kaushansky K. Thrombopoietin-induced activation of the mitogen-activated protein kinase (MAPK) pathway in normal megakaryocytes: role in endomitosis. Blood 1999;94:1273-82

35. Erickson-Miller C, Delorme E, Tian SS, et al. Preclinical activity of eltrombopag (SB-497115), an oral, non-peptide thrombopoietin Receptor Agonist. Stem Cells; In press

•• **A summary of the preclinical studies demonstrating the selective interaction of eltrombopag with the TpoR and the effects of eltrombopag on megakaryocyte differentiation in platelets.**

36. Us Food and Drug Administration. 2008 Center for Drug Evaluation and Research (CDER) Meeting Documents. Oncologic Drugs Advisory Committee May 30, 2008. Available from: http://wwwfdagov/ohrms/dockets/AC/cder08html [Last accessed 15 October 2008]

37. Jenkins JM, Williams D, Deng Y, et al. Phase 1 clinical study of eltrombopag, an oral, nonpeptide thrombopoietin receptor agonist. Blood 2007;109:4739-41

•• **An investigation of the pharmacology of eltrombopag in healthy volunteers.**

38. Bussel JB, Cheng G, Saleh MN, et al. Eltrombopag for the treatment of chronic idiopathic thrombocytopenic purpura. N Engl J Med 2007;357:2237-47

•• **A randomized, double-blind, placebo-controlled trial that showed the efficacy and safety of short-term treatment with eltrombopag.**

39. Bussel J, Provan A, Shamsi T, et al. Eltrombopag raises platelet count and reduces bleeding compared with placebo during short-term treatment in chronic idiopathic thrombocytopenic purpura: a phase III study [abstract 0390]. Haematologica 2007;92(Suppl 1)

• **An analysis of the efficacy results of short-term treatment with eltrombopag in the TRA100773B trial.**

40. Bussel JB, Cheng G, Saleh M, et al. Long-term safety and efficacy of oral eltrombopag for the treatment of idiopathic thrombocytopenic purpura (ITP) [abstract 0949]. Haematologica 2008;93

•• **A preliminary analysis of adverse events and continued response during long-term dosing of eltrombopag.**

41. Psaila B, Bussel JB, Vasey S, et al. Efficacy and safety of repeated intermittent treatment with eltrombopag in patients with chronic ITP [abstract 0294]. Haematologica 2008;93

•• **A preliminary analysis of response patterns in patients with chronic ITP upon repeated administration of eltrombopag.**

42. Mchutchison JG, Dusheiko G, Shiffman ML, et al. Eltrombopag for thrombocytopenia in patients with cirrhosis associated with hepatitis C. N Engl J Med 2007;357:2227-36

43. Gernsheimer T. Epidemiology and pathophysiology of immune thrombocytopenic purpura. Eur J Haematol Suppl 2008;3-8

44. George JN. Management of patients with refractory immune thrombocytopenic purpura. J Thromb Haemost 2006;4:1664-72

45. Armstrong GL, Wasley A, Simard EP, et al. The prevalence of hepatitis C virus infection in the United States, 1999 through 2002. Ann Intern Med 2006;144:705-14

46. Sulkowski MS. Management of the hematologic complications of hepatitis C therapy. Clin Liver Dis 2005;9:601-16

47. Bashour FN, Teran JC, Mullen KD. Prevalence of peripheral blood cytopenias (hypersplenism) in patients with nonalcoholic chronic liver disease. Am J Gastroenterol 2000;95:2936-9

48. Roberts SA. High-throughput screening approaches for investigating drug metabolism and pharmacokinetics. Xenobiotica 2001;31:557-89

49. Schwardt O, Kolb H, Ernst B. Drug discovery today. Curr Top Med Chem 2003;3:1-9

50. Muegge I. Selection criteria for drug-like compounds. Med Res Rev 2003;23:302-21

51. Rees DC, Congreve M, Murray CW, Carr R. Fragment-based lead discovery. Nat Rev 2004;3:660-72

52. Scheuermann J, Dumelin CE, Melkko S, Neri D. DNA-encoded chemical libraries. J Biotechnol 2006;126:568-81

53. Kaushansky K. Hematopoietic growth factor mimetics. Ann NY Acad Sci 2001;938:131-8

54. Cwirla SE, Balasubramanian P, Duffin DJ, et al. Peptide agonist of the thrombopoietin receptor as potent as the natural cytokine. Science 1997;276:1696-9

55. de Serres M, Ellis B, Dillberger JE, et al. Immunogenicity of thrombopoietin mimetic peptide GW395058 in BALB/c mice and New Zealand white rabbits: evaluation of the potential for thrombopoietin neutralizing antibody production in man. Stem Cells 1999;17:203-9

56. Wang B, Nichol JL, Sullivan JT. Pharmacodynamics and pharmacokinetics of AMG 531, a novel thrombopoietin receptor ligand. Clin Pharmacol Ther 2004;76:628-38

57. Roopenian DC, Akilesh S. FcRn: the neonatal Fc receptor comes of age. Nat Rev Immunol 2007;7:715-25

Affiliation

Roberto Stasi
Regina Apostolorum Hospital,
Department of Medical Sciences,
Via S Francesco, 50, 00041 Albano Laziale, Italy
Tel: +01139 06 932989;
Fax: +01139 06 233231809;
E-mail: roberto.stasi@uniroma2.it