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Effect of the nonpeptide thrombopoietin receptor agonist eltrombopag on megakaryopoiesis of patients with lower risk myelodysplastic syndrome

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ABSTRACT

Eltrombopag is a nonpeptidyl thrombopoietin receptor agonist. We evaluated the *ex vivo* effect of eltrombopag on megakaryopoiesis of patients with lower risk myelodysplastic syndromes (MDSs). At a concentration of 0.1 μg/mL, eltrombopag resulted in a significant increase in the number of megakaryocytic colonies in MDS patients and healthy controls compared to baseline. This dose of eltrombopag did not exert any significant change in the proliferation rate or the survival characteristics of patient CD34$^+$ cells that might clinically imply an unfavorable effect on patients' outcome. These encouraging preclinical data support the rationale of using eltrombopag in the clinic for alleviation of thrombocytopenia in lower risk MDS patients.

1. Introduction

Thrombocytopenia is a serious problem in patients with myelodysplastic syndromes (MDSs) as it may result in shortened survival and impaired quality of life due to bleeding complications. Its frequency appears to be associated with certain karyotypic abnormalities [1] and the international prognostic scoring system (IPSS) risk with an increased prevalence in higher risk groups [2]. In patients with lower IPSS, the frequency of thrombocytopenia ranges from 5% to 40% and may occur as a single abnormality or in conjunction with other cytopenias [3]. The pathophysiologic mechanisms underlying thrombocytopenia involve mainly ineffective bone marrow (BM) megakaryopoiesis due to impaired proliferation and differentiation of megakaryocytes and their precursors in association with increased programmed cell death attributed to both intrinsic cellular defects and microenvironmental inhibitory effects [4,5]. Increased peripheral platelet destruction due to (non-)immune mechanisms may also have a role [4,6].

Management of thrombocytopenia in MDS remains a challenge as most of the available pharmacologic agents may, *per se,* cause or worsen this abnormality [2]. There is currently an emerging interest in exploring the use of the second generation thrombopoietin receptor (TpoR) agonists eltrombopag and romiplostim in the treatment of MDS patients with thrombocytopenia [7,8]. Although the safety and efficacy of these agents have been clearly shown for patients with refractory chronic immune thrombocytopenic purpura (ITP) [9,10], thorough preclinical studies are necessary to determine their effect in MDS before their introduction in the clinic. Recently, it was shown that eltrombopag may effectively increase megakaryocytic differentiation and colony formation *ex vivo* in patients with acute myeloid leukemia (AML) and high risk MDS without inducing blast cell proliferation or expansion [7].

In the current study we investigate for the first time the *ex vivo* effect of eltrombopag on megakaryopoiesis of patients with lower risk MDS aiming to provide the prerequisite experimental data for the clinical use of the agent in this group of patients. We have specifically investigated the *ex vivo* effect of different concentrations of eltrombopag on megakaryocytic progenitor cell survival characteristics and colony growth as well as its action on blast cell proliferation.

2. Design and methods

2.1. BM samples

BM cells obtained from posterior iliac crest aspirates of 18 MDS patients with low/intermediate-1 IPSS score (Table 1) and 5 hematologically healthy subjects, were diluted 1:1 in Iscove Modified Dulbeco Medium (IMDM; Gibco, Invitrogen, UK), supplemented with 10 IU/mL preservative-free heparin (Sigma, St. Louis, MO) and 100 IU/mL penicillin–streptomycin (PS; Gibco). Diluted BM samples were centrifuged on Lymphoprep (Nycomed Pharma, Oslo, Norway) at 400 × *g* for 30 min

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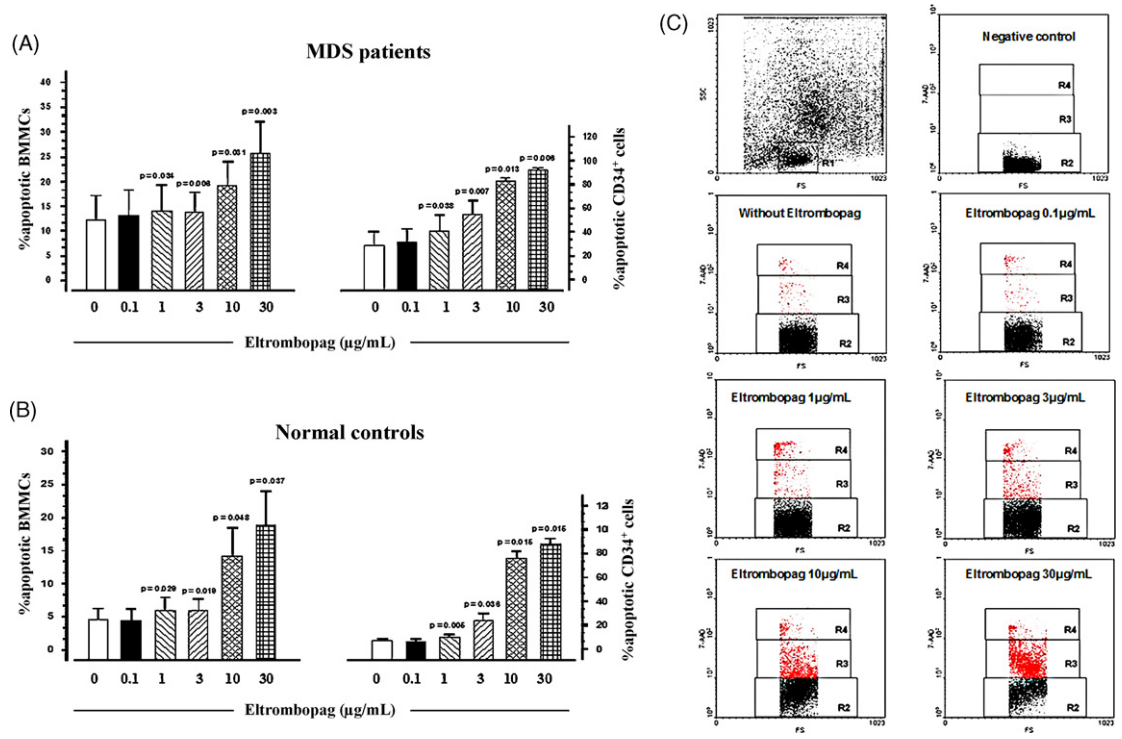

Fig. 1. The effect of eltrombopag on the survival characteristics of BMMCs and CD34$^+$ cells. BMMCs or purified CD34$^+$ cells from MDS patients ($n=5$) and healthy controls ($n=4$) were stained with 7-amino-actinomycin D (7AAD) following overnight incubation with different concentrations of eltrombopag, for the evaluation of the proportion of apoptotic cells by flow cytometry. The bars in graphs (A) and (B) represent the mean proportion of total apoptotic (7AAD^{dim} plus 7AAD^{bright}) BMMCs and CD34$^+$ cells in patients and controls, respectively. Error bars represent the standard error of the mean (SEM). Analysis between eltrombopag treated and untreated cultures has been performed by means of the paired *t*-test and statistically significant *P* values are indicated. The dot plots in graph (C) depict the results from the flow cytometric analysis of apoptosis of BMMCs from a representative MDS patient. All analyses have been performed in the gate of cells with low FSC/SSC properties where the BMMCs are included (R1). Negative control represents cells with no 7AAD. Gates R2, R3 and R4 show the proportion of 7AAD$^-$ (live), 7AAD^{dim} (early apoptotic) and 7AAD^{bright} (late apoptotic), respectively in the presence or absence of different concentrations of eltrombopag as shown.

at room temperature to obtain the BM mononuclear cells (BMMCs). Institutional ethics committee approval was granted prior to the study and informed consent was provided by all subjects studied.

2.2. Reagents

Eltrombopag (SB-497115) was kindly provided by GlaxoSmithKline (Collegeville, PA). The reagent was dissolved in ddH_2O at various concentrations (1 mg/mL, 100 μg/mL, 20 μg/mL, 2 μg/mL, 1 μg/mL) and stored light protected at room temperature, under constant stirring for up to 3 weeks.

2.3. Purification of CD34$^+$ cells

CD34$^+$ early progenitor cells were isolated from BMMCs by immunomagnetic labeling and sorting according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany) [11]. In all experiments, purity of CD34$^+$ cells was above 90% as estimated by flow cytometry.

2.4. Short-term cultures

We cultured 1×10^6 BMMCs or 0.1×10^6 CD34$^+$ cells in IMDM/1% fetal bovine serum (FBS; Gibco) culture medium in the presence or absence of various

Table 1
Clinical and laboratory data of MDS patients studied.

UPN	Age/sex	WHO subtype	IPSS risk category	WBC ($\times 10^9$/L)	Neutro ($\times 10^9$/L)	Hgb (g/dL)	Plts ($\times 10^9$/L)	Cytogenetics
1	85/M	RCMD	Low	5.4	4.1	10.7	71	46, XY
2	80/M	RCMD	Int-1	4.2	1.7	9	65	46,XY
3	57/M	RAEB-1	Int-1	4.5	2.5	11.0	89	46, XY
4	67/M	RAEB-1	Int-1	4.5	2.5	9.9	87	46, XY
5	71/M	RCMD	Low	2.1	0.4	12.8	99	46, XY
6	81/M	RA	Int-1	4.4	2.5	11.3	95	46, XY
7	68/M	RCMD	Low	5.1	3.5	11.1	35	46, XY
8	64/M	RCMD	Int-1	10	5.8	12	289	47, XXY_C, −Y, +14
9	56/M	RCMD	Low	8.6	2.4	11	275	46, XY
10	79/M	RA	Low	7.5	5.5	9.8	318	46, XY
11	52/F	RA	Low	8.6	2.4	11	275	46, XX
12	79/F	RAEB-1	Int-1	2.2	1.0	8.5	205	46, XX
13	71/M	Del(5q)	Low	6.5	3.6	7.3	358	46, XY, del(5q)
14	82/M	RA	Low	4.7	2.7	10.9	135	46, XY
15	61/F	RA	Low	6.5	3.0	11.4	207	46, XX
16	78/F	RA	Low	5.9	3.2	10.9	251	46, XX
17	74/M	RA	Low	5.2	2.8	10.7	197	46, XY
18	84/M	RA	Low	8.2	6.3	11.4	188	46, XY

Abbreviations: MDS, myelodysplastic syndrome; UPN, unique patient number; WHO, World Health Organization; IPSS, international prognostic scoring system; RCMD, refractory cytopenia with multilineage dysplasia; RA, refractory anemia; RAEB, RA with excess of blasts; Int-1, intermediate-1; Del(5q), deletion of the long arm of chromosome 5.

concentrations of eltrombopag (ranging from 0.01 μg/mL to 30 μg/mL) for 12, 48, 72 h in a 37 °C-5% CO_2 fully humidified atmosphere. Cells were then used for flow cytometry and apoptosis assays.

2.5. Flow cytometric analysis of BM megakaryocytic progenitor cells

An indirect immunofluorescence technique was used to quantitate the expression of CD61 (integrin beta3; Glycoprotein IIIα, GpIIIα) surface antigen on cells treated or not with eltrombopag as described above. In brief, 0.1×10^6 $CD34^+$ cells were stained with anti-CD34-phycoerythrin (PE) (QBEND-10; Beckman Coulter Inc., Fullerton, CA) and anti-CD61-fluorescein isothiocyanate (FITC) (SZ21; Beckman Coulter) mouse antihuman monoclonal antibodies (mAb) or the IgG isotype-matched controls for 30 min on ice. Cells were washed twice in phosphate-buffered saline (PBS)/1% FBS/0.05% sodium azide and fixed in 200 μL 2% paraformaldeyde solution (PFA; Sigma). Data were acquired and processed in an Epics Elite model flow cytometer (Coulter, Miami, FL). The proportion of the $CD61^+$ megakaryocytic progenitor cells was evaluated in the gate of $CD34^+$ cells.

2.6. Apoptosis assay with 7-amino-actinomycin D staining

0.5×10^6 BMMCs or 0.1×10^6 purified $CD34^+$ were stained with 40 μL 7-amino-actinomycin D (7AAD; Calbiochem–Novabiochem, La Jolla, CA) solution (200 μg/mL), as previously described [12]. Data were analyzed using three parameters: forward scatter (FSC), side scatter (SSC) and 7AAD immunofluorescence. A scattergram was created by combining FSC with 7AAD fluorescence to quantitate $7AAD^-$ (live), $7AAD^{dim}$ (early apoptotic), and $7AAD^{bright}$ (late apoptotic) cells in the gate of cells with low FSC/SSC properties or in the gate of $CD34^+$ cells [11,13]. Results of apoptosis were expressed as total $7AAD^+$ ($7AAD^{dim}$ plus $7AAD^{bright}$) cells. A representative example from flow cytometric analysis is shown in Fig. 1.

2.7. Megakaryocytic progenitor cell assays

For the characterization and quantification of the megakaryocyte colony-forming units (CFU-Meg), we cultured 0.5×10^6 BMMCs per chamber in a double-chamber slide in the presence or absence of different concentrations of eltrombopag (ranging from 0.01 μg/mL to 30 μg/mL) using a collagen-based commercially available semisolid culture medium (MegaCult-C, StemCell Technologies, Vancouver, BC, Canada) supplemented with 50 ng/mL recombinant human (rh) Tpo, 10 ng/mL rh interleukin-3 and 10 ng/mL rh interleukin-6, according to the manufacturer's instructions. Following a 12-day incubation at 37 °C in 5% CO_2 humidified atmosphere, colonies were scored after fixation and staining of culture slides with anti-CD41 (GpIIb/IIIα) mAb (5B12; Dako, Glostrup, Denmark) using the alkaline phosphatase anti-alkaline phosphatase technique (APAAP), as previously described [14,15]. Colonies were microscopically identified and scored as pure CFU-Meg containing only megakaryocytes, mixed CFU-Meg containing megakaryocytes and non-megakaryocytic cells, and colonies non-CFU-Meg containing cells other than megakaryocytes. Pure CFU-Meg was classified according to the size and maturity as small (late) and large (early) CFU-Meg consisting of less and more than 50 cells per colony, representing colonies derived from mature and immature progenitor cells, respectively. Results were also expressed as total CFU-Meg, which included pure and mixed megakaryocytic colonies.

2.8. Methyl Triazol Tetrazolium (MTT) assay

Immunomagnetically sorted $CD34^+$ cells from MDS patients were cultured in 96-well plates at a concentration of 10^4 cells/well in triplicate for seven consecutive days in the presence or absence of 0.1 μg/mL eltrombopag. Every day cells were incubated for 4 h with MTT (Sigma) solution (1 mg/mL in PBS). Following dilution of the produced formazan with equal volume of isopropanol, the optical density was measured at 630 nm to obtain a curve of absorbance corresponding to the number of live cells versus the days of culture

2.9. Statistical analysis

Data were analyzed in the GraphPAd Prism statistical PC program (GraphPad Software, San Diego, CA) and expressed as mean ± standard deviation (SD). The Student's *t*-test for pairs (paired *t*-test) was used to compare individual values derived from cultures treated or not with eltrombopag. The two-way analysis of variance (two-way ANOVA) was applied to compare cell viability in various time points between eltrombopag treated and untreated cultures.

3. Results

3.1. Effect of eltrombopag on the survival characteristics of BMMCs and $CD34^+$ cells

We initially evaluated the effect of eltrombopag on the survival characteristics of BMMCs and immunomagnetically sorted $CD34^+$ cells from MDS patients ($n = 5$) and healthy controls ($n = 4$) after 12-h (overnight) incubation with concentrations ranging from 0.1 μg/mL to 30 μg/mL. Results are shown in Fig. 1. In patient cultures the presence of eltrombopag at a concentration of 1 μg/mL, 3 μg/mL, 10 μg/mL and 30 μg/mL resulted in a dose-dependent increase in the proportion of apoptotic BMMCs (13.94 ± 12.21%, 13.76 ± 11.32%, 19.16 ± 10.66%, 25.72 ± 14.25%, respectively) compared to baseline (12.24 ± 11.08%) ($P = 0.034$, $P = 0.006$, $P = 0.031$, $P = 0.003$, respectively) (Fig. 1A). Similarly, in normal cultures the above concentrations of eltrombopag resulted in an increase in the proportion of apoptotic BMMCs (5.85 ± 4.06%, 5.98 ± 3.50%, 14.20 ± 8.49%, 18.85 ± 10.27%, respectively) compared to baseline (4.57 ± 3.45%) ($P = 0.029$, $P = 0.019$, $P = 0.048$, $P = 0.037$, respectively) (Fig. 1B). In the presence of the above concentrations of eltrombopag, a dose-dependent increase was also obtained in the proportion of apoptotic $CD34^+$ cells in patients (40.44 ± 30.30%, 54.78 ± 25.71%, 82.74 ± 5.70%, 92.34 ± 3.34%, respectively) ($P = 0.038$, $P = 0.007$, $P = 0.013$, $P = 0.006$, respectively) and healthy controls (10.07 ± 4.20%, 23.85 ± 11.56%, 76.42 ± 10.36%, 87.92 ± 9.18%, respectively) ($P = 0.005$, $P = 0.036$, $P = 0.015$, $P = 0.015$, respectively) in comparison to baseline (28.62 ± 26.28% in patients and 6.67 ± 4.27% in the controls) (Fig. 1A and B). The concentration of 0.1 μg/mL eltrombopag however, did not result in any significant increase in the proportion of apoptotic BMMCs or $CD34^+$ cells in either patients (12.98 ± 11.84% and 30.92 ± 25.75%, respectively) or healthy controls (4.42 ± 3.47% and 6.02 ± 4.64%, respectively) compared to untreated cultures (Fig. 1A and B). In keeping with these finding, the dose 0.1 μg/mL eltrombopag did not change significantly the proportion of $CD61^+$ megakaryocytic progenitor cells within the $CD34^+$ cell fraction in patients (20.1 ± 8.14%) or healthy controls (20 ± 9.72%) compared to baseline (21.5 ± 8.39% and 19.57 ± 10.25%, respectively).

Having demonstrated that the dose of 0.1 μg/mL eltrombopag does not affect the survival of BMMCs or $CD34^+$ cells of healthy subjects or MDS patients, we evaluated the prolonged effect of this concentration on the survival characteristics of MDS-derived cell populations ($n = 5$) following incubation for 12, 48 and 72 h. The presence of eltrombopag did not result in any significant increase in the proportion of apoptotic BMMCs (4.98 ± 2.32%, 4.66 ± 1.26% and 5.9 ± 4.03%, respectively) or $CD34^+$ (5.96 ± 2.89%, 7.74 ± 3.80% and 9.34 ± 4.09%, respectively) compared to untreated cultures (4.26 ± 1.95%, 4.44 ± 1.23%, 5.32 ± 3.51%, respectively and 5.02 ± 1.70%, 6.92 ± 2.68%, 8.68 ± 3.14%, respectively) at any time-point tested, further demonstrating that this dose of eltrombopag retains the cellular survival characteristics.

3.2. Effect of eltrombopag on the clonogenic potential of megakaryocytic progenitor cells

Using a semisolid culture system containing collagen and a combination of thrombopoiesis-stimulating cytokines, we initially evaluated the CFU-Meg colony formation in the presence or absence of eltrombopag at concentrations ranging from 0.1 μg/mL to 30 μg/mL in MDS patients ($n = 18$) and healthy controls ($n = 5$). In the patients, the dose of 1 μg/mL resulted in a decrease in the total CFU-Meg number (155 ± 109 CFU-Meg per 0.5×10^5 BMMCs) compared to baseline (196 ± 157 CFU-Meg per 0.5×10^5 BMMCs) although not at a statistically significant level ($P = 0.0535$) (Fig. 2A). In accordance with the survival data, the higher concentrations of eltrombopag, i.e. 3 μg/mL, 10 μg/mL and 30 μg/mL resulted in a significant decrease in colony numbers (140 ± 109 CFU-Meg, 115 ± 109 CFU-Meg and 74 ± 75 CFU-Meg per 0.5×10^5, respectively) compared to untreated cultures ($P = 0.0107$, $P = 0.0022$ and $P = 0.0001$, respectively) (Fig. 2A). The decrease concerned all CFU-Meg types, i.e. the small (derived from mature megakaryocytic progenitor cells), large (derived from immature megakaryocytic

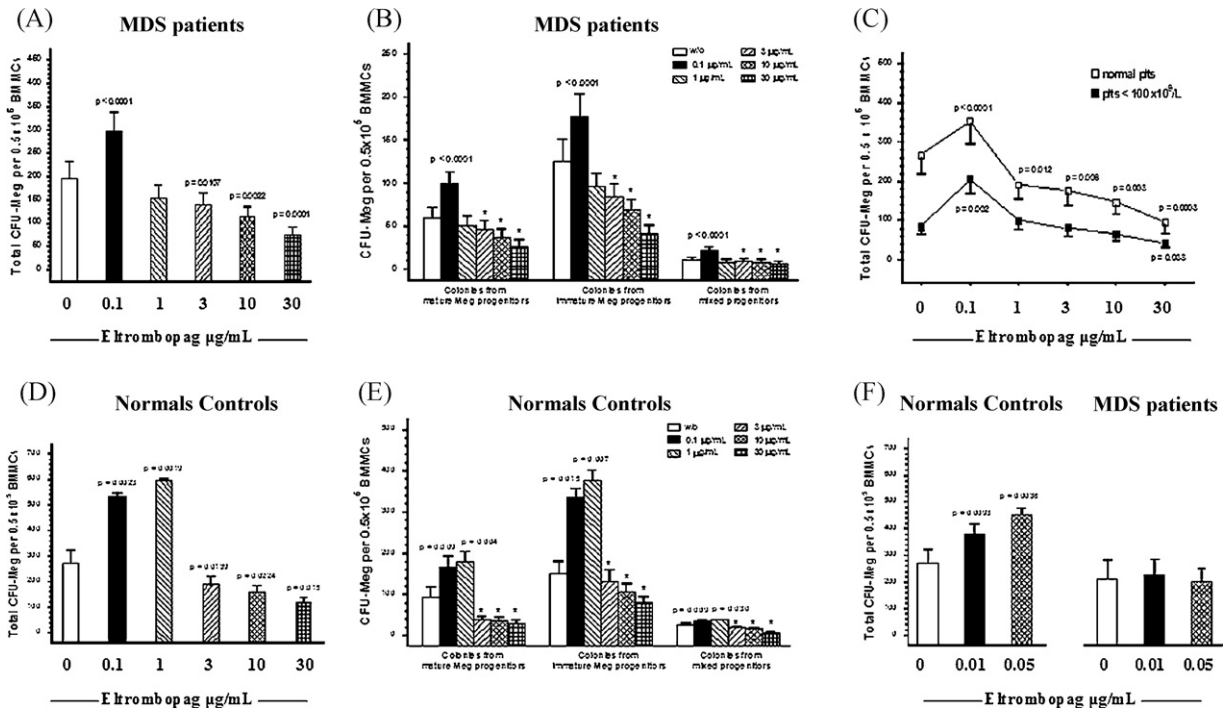

Fig. 2. The effect of eltrombopag on the clonogenic potential of megakaryocytic progenitor cells. BMMCs from MDS patients ($n=18$) and healthy controls ($n=5$) were cultured in a collagen-based culture medium appropriately supplemented with thrombopoiesis-inducing cytokines, in the presence or absence of different concentrations of eltrombopag. Megakaryocyte colony-forming units (CFU-Meg) were enumerated after 12 days of culture. The bars in graphs (A) and (D) represent the mean number of total CFU-Meg obtained in MDS patients and healthy controls, respectively. Error bars represent the standard error of the mean (SEM). Comparison between eltrombopag treated and untreated cultures has been performed by means of the paired *t*-test and only statistically significant *P* values are indicated. The bars in graphs (B) and (E) represent the mean (+SEM) differential colony numbers derived from mature, immature and mixed megakaryocytic progenitor cells in MDS patients and healthy controls, respectively. The *P* values show the statistically significant increase in CFU-Meg numbers in the presence of the indicated dose of eltrombopag compared to baseline (paired *t*-test). Asterisk (*) denotes the statistical significant decrease ($P<0.05$) in CFU-Meg number in cultures treated with the indicated concentration of eltrombopag compared to untreated cultures (paired *t*-test). (C) The open and solid symbols depict the mean (+SEM) total CFU-Meg numbers in MDS patients with normal and low ($<100\times10^9$/L) platelet counts, respectively, in cultures treated with different concentrations of eltrombopag. Only statistically significant *P* values obtained from comparisons between eltrombopag treated and untreated cultures (paired *t*-test) are indicated. (F) The bars represent the mean (+SEM) total CFU-Meg numbers obtained in MDS patients ($n=7$) and healthy controls in the presence or absence of the indicated low concentrations of eltrombopag. The *P* values indicated the statistically significant differences between cultures treated with eltrombopag and baseline (paired *t*-test).

progenitor cells) and mixed (derived from early progenitors) colonies (Fig. 2B). In the presence of 0.1 μg/mL eltrombopag, however, a statistically significant increase was obtained in the total CFU-Meg (296 ± 175 CFU-Meg per 0.5×10^5 BMMCs) compared to baseline numbers ($P<0.0001$) (Fig. 2A). This increase was due to a parallel improvement of the small (99 ± 61 CFU-Meg per 0.5×10^5), large (177 ± 112 CFU-Meg per 0.5×10^5) and mixed (22 ± 16 CFU-Meg per 0.5×10^5) colonies compared to baseline (60 ± 50 CFU-Meg, 125 ± 107 CFU-Meg and 10 ± 14 CFU-Meg per 0.5×10^5, respectively) ($P<0.0001$, $P<0.0001$ and $P<0.0001$, respectively) (Fig. 2B).

Having demonstrated the beneficial effect of 0.1 μg/mL eltrombopag in the clonogenic potential of megakaryocytic progenitor cells in MDS patients, we performed an extra analysis based on patient platelet counts (Fig. 2C). In the group of patients with low platelets ($<100\times10^9$/L, $n=7$), the above dose of eltrombopag resulted in a 2.5-fold increase in the total CFU-Meg number (205 ± 98 CFU-Meg per 0.5×10^5) compared to baseline (83 ± 45 CFU-Meg per 0.5×10^5, $P=0.0021$) with a significant increase in the small, large and mixed colonies ($P=0.0078$, $P=0.0025$ and $P<0.0001$, respectively). In the group of patients with normal platelets ($n=11$) the dose of 0.1 μg/mL eltrombopag resulted in a 1.3-fold increase in the total CFU-Meg number (353 ± 192 CFU-Meg per 0.5×10^5) compared to baseline (267 ± 162 CFU-Meg per 0.5×10^5, $P<0.0001$) associated with a significant increase in the small, large and mixed colonies ($P<0.0001$, $P<0.0001$ and $P<0.0001$, respectively). Higher concentration of eltrombopag resulted in a decrease in CFU-Meg colony numbers in both groups of patients.

We next evaluated the effect of eltrombopag in the group of healthy controls ($n=5$). In agreement with the patient data, the concentrations of 3 μg/mL, 10 μg/mL and 30 μg/mL resulted in a significant decrease in the total CFU-Meg numbers (188 ± 75 CFU-Meg, 160 ± 54 CFU-Meg and 120 ± 41 CFU-Meg per 0.5×10^5 BMMCs, respectively) compared to baseline (269 ± 117 CFU-Meg per 0.5×10^5 BMMCs) ($P=0.0139$, $P=0.0024$ and $P=0.015$, respectively) due to parallel decrease in all CFU-Meg colony types (Fig. 2D and E). A significant improvement however, was obtained following incubation with 0.1 μg/mL and 1 μg/mL eltrombopag (535 ± 28 CFU-Meg and 593 ± 24 CFU-Meg per 0.5×10^5 BMMCs, respectively) in comparison to baseline ($P=0.0023$ and $P=0.0019$, respectively) that was associated with a significant increase in all CFU-Meg colony types (Fig. 2D and E).

Finally, we evaluated the *ex vivo* effect of lower concentrations of eltrombopag i.e. 0.01 μg/mL and 0.05 μg/mL, in the patients (3 with low and 4 with normal platelets from the original group) and control subjects (Fig. 2F). In the group of controls, even the lower concentrations of eltrombopag increased significantly the total CFU-Meg numbers (381 ± 81 CFU-Meg and 452 ± 58 CFU-Meg per 0.5×10^5 BMMCs, respectively) in comparison to untreated cultures ($P=0.0093$ and $P=0.0036$, respectively) and the increase was associated with a significant improvement in all CFU-Meg subtypes. In the group of patients, although the lower concentrations of eltrombopag increased the CFU-Meg numbers compared to baseline, the difference obtained was not statistically significant.

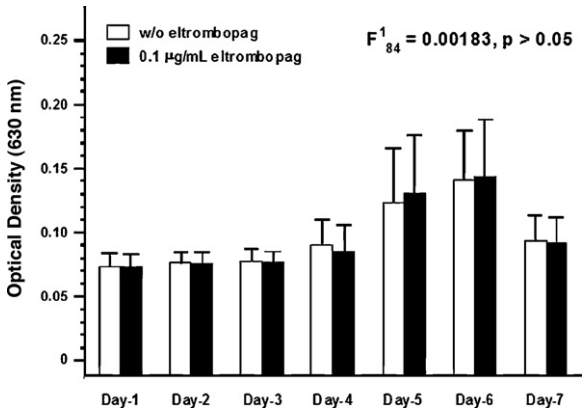

Fig. 3. The effect of eltrombopag on the growth of CD34⁺ cells from MDS patients. Immunomagnetically sorted CD34⁺ cells from MDS patients ($n = 7$) were cultured for 7 days in the presence or absence of eltrombopag (0.1 μg/mL) and the rate of cell growth was evaluated by the Methyl Triazol Tetrazolium (MTT) assay. The bars represent the mean optical density at 630 nm corresponding to the number of live cells in eltrombopag treated and untreated cultures. The error bars represent the standard error of the mean (SEM). Comparison between eltrombopag treated and untreated cultures through the 7-day culture period was performed by means of the two-way analysis of variance. The obtained F value did not demonstrate statistically significant difference.

Taken together, all the above data clearly show that eltrombopag at a dose of 0.1 μg/mL displays a beneficial effect on the clonogenic potential of megakaryocytic progentitor cells in healthy subjects and MDS patients with low or normal platelet counts.

3.3. *Effect of eltrombopag on the proliferation rate of CD34⁺ cells*

As TpoR is expressed not only on normal megakaryocytes, platelets and hematopoietic progenitors but also on MDS blast cells [16,17], we investigated whether the clonogenic-inducing dose of eltrombopag (0.1 μg/mL) might also exert a proliferative effect on patient blast cells. We therefore evaluated the proliferation rate of immunomagnetically sorted CD34⁺ cells from MDS patients ($n = 7$) following incubation with 0.1 μg/mL eltrombopag using the MTT assay (Fig. 3). No statistically significant difference was identified in cell proliferation rate between treated and untreated cultures over the 7-day culture period ($F^1_{84} = 0.00183$, $P > 0.05$) suggesting that the megakaryopoiesis-inducible effect of eltrombopag in MDS patients is not associated with increased risk for blast cell proliferation.

4. Discussion

Eltrombopag, a nonpeptidyl small molecule belonging to the second generation TpoR agonists, has been approved for the treatment of chronic ITP and has been also proved effective in thrombocytopenia related to hepatitis C infection [9,18–20]. *In vitro* studies have shown that eltrombopag interacts selectively with TpoR and activates intracellular signal transduction pathways additively and not competitively with Tpo, leading to increased megakaryocytic progenitor cell proliferation, differentiation, and ultimately platelet production [21]. Thrombocytopenia associated with ineffective BM platelet production in patients with MDS represents a potential therapeutic target for eltrombopag [22,23]. This possibility remains to be proven in the setting of clinical trials but has been recently investigated in patients with high risk MDS *ex vivo* [7].

In the current study we examined for the first time the *ex vivo* effect of eltrombopag on the survival, proliferation and differentiation of hematopoietic and megakaryocytic progenitor cells in patients with lower risk MDS. We found that eltrombopag at a concentration of 0.1 μg/mL resulted in an induction of patients' megakaryocytic progenitor cell growth. This dose of eltrombopag displayed a positive effect on megakaryopoiesis irrespective of patient platelet counts and resulted in a significant increase in the clonogenic potential of cells of all stages of megakaryopoiesis from the mixed, to the immature and the more mature megakaryocytic progenitor cells. These data might clinically imply an increased platelet production in the patients. The higher concentrations of eltrombopag, i.e. 3–30 μg/mL, resulted in a significant decrease in the clonogenic potential of patient megakaryocytic progenitor cells whereas the dose of 1 μg/mL did not exert any significant effect in patients' colony growth. Similarly, the lower doses, i.e. 0.01 μg/mL and 0.05 μg/mL, did not result in any significant effect in megakaryocytic colony growth. In the healthy controls, a dose-dependent increase in the clonogenic potential of BM megakaryocytic progenitor cells was obtained with concentrations of eltrombopag ranging from 0.01 μg/mL to 1 μg/mL that involved all stages of megakaryocytic development. Consistent with the patient data, concentrations of eltrombopag ranging from 3 μg/mL to 30 μg/mL resulted in significant reduction in the clonogenic potential of megakaryocytic progenitor cells of the healthy controls. The inhibitory effect of high concentrations of eltrombopag on cell growth has been also shown in the study of Will et al. [7]. However, in contrast to our study, the concentration of 3 μg/mL was shown to induce the clonogenic potential of megakaryocytic progenitor cells in both AML/MDS patients and healthy controls [7]. Results however are not absolutely comparable between the two studies because in the study of Will et al., colony data were normalized according to control data derived from Tpo-treated cultures. Nonetheless, the inhibitory effect of higher concentrations of eltrombopag might be attributed to the implication of different signal transduction pathways under the influence of different concentrations of the compound. Furthermore, in a recent study it was shown that eltrombopag at a concentration higher than 2 μg/ml resulted in a decrease in proliferation of leukemic and Tpo-dependent cell lines and an increase in the necrotic/dead cell population even 2 h after incubation with the compound, thus implying a possible involvement of eltrombopag in a mechanism other that apoptosis such as autophagy or entosis [24]. However, such speculations need further investigation.

Previous studies have shown that TpoR may be expressed on blast cells [16,17] and BMMCs [25] of patients with MDS. It has been also reported that Tpo may stimulate the proliferation of blasts cells [16] and BMMCs [25] from MDS patients, particularly those belonging to the higher risk groups. Preclinical *in vitro* studies have shown that eltrombopag results in activation of some of the intracellular signaling pathways similar to those induced by Tpo, although with lesser intensity [21,26]. Accordingly, the possibility of induction of malignant cell growth by eltrombopag is an issue that needs to be investigated in MDS patients. Indeed, recent clinical data have shown that treatment of MDS patients with the TpoR agonist romiplostim may transiently increase the BM blast cells, an effect however, that seems to be normalized after the drug withdrawal [8]. In our study we examined the *in vitro* effect of eltrombopag on the survival characteristics of BMMCs and CD34⁺ cells as well as on the proliferation rate of blast cells. We found that the eltrombopag at concentration 1–30 μg/mL significantly decreased the survival of BMMCs and CD34⁺ from MDS patients and healthy controls whereas the concentration of 0.1 μg/mL did not exert any significant effect in the survival characteristics of the above cell populations even following prolonged incubation. These data in association with the colony data provide further evidence that the optimum concentration of eltrombopag that might induce *in vivo* megakaryopoiesis in MDS patients without any potential negative effect on the survival of BMMCs or CD34⁺ cells is that of 0.1 μg/mL. Specifically, using this concentration, we found evidence for neither

increased apoptosis of BMMCs, nor for reduction of apoptotic activity of $CD34^+$ blast cell population, in any of the patients studied that might result in an unfavorable effect on patients' outcome. Furthermore, using this concentration of eltrombopag we did not observe any significant change in the proliferation rate of patient blast cells that might clinically imply an increase risk for AML transformation. Our findings are in agreement with the data of Will et al. who also showed that eltrombopag does not stimulate the blast cell growth and proliferation in patients with AML/high risk MDS [7]. This differential *in vitro* effect between eltrombopag and Tpo in regards to blast cell proliferation might be related to the different binding sites of these agents in the TpoR that probably result in differences in their intracellular mechanisms of action. Indeed, differences between the two agents have been described in the activation intensity of the signaling transducers and activators of transcription (STAT) in megakaryocytic cell lines [21] and also in the capacity of activating the Akt pathway in platelets [26].

In summary, our results show that eltrombopag at levels that can be easily reached by once daily administration according to the available pharmacokinetic data, where a plasma maximum concentration 7.3 μg/mL has been reported at approximately 2.5–5 h after the dose of 75 mg [27], may stimulate all stages of megakaryopoiesis and may therefore induce platelet production in patients with lower risk MDS without any risk for blast cell proliferation. These encouraging *in vitro* data support the rationale of using eltrombopag in the clinic for alleviation of thrombocytopenia in this group of patients. The anticipated beneficial effect remains to be confirmed in the setting of clinical trials.

Conflict of interest statement

The authors declare that they have no financial or personal relationships with other people or organizations that could inappropriately influence (bias) their work.

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Contributions. I.M. performed most of the experiments and contributed to the writing of the paper; K.P., K.P. and M.P. performed experiments, provided bone marrow samples and analyzed data; C.K. and C.P. provided bone marrow samples and reviewed the manuscript; H.A.P. designed and supervised the study, analyzed and interpreted the data and wrote the paper.

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