AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy



# Oxazolidinone antibiotics impair *ex vivo* megakaryocyte differentiation from hematopoietic progenitor cells and their maturation into platelets

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**ABSTRACT** Oxazolidinones (linezolid and tedizolid) adverse reactions include thrombocytopenia, the mechanism of which is still largely unknown. In cultured cells, oxazolidinones impair mitochondrial protein synthesis and oxidative metabolism. As mitochondrial activity is essential for megakaryocyte differentiation and maturation into platelets, we examined whether oxazolidinones impair these processes *ex vivo* and alter, in parallel, the activity of mitochondrial cytochrome *c*-oxidase (CYTOX; enzyme partly encoded by the mitochondrial genome) and cell morphology. Human CD34+ cells were isolated, incubated with cytokines (up to 14 days) and clinically relevant oxazolidinone concentrations or in control conditions, and used for (i) clonogenic assays [counting of megakaryocyte (CFU-Mk), granulocyte-monocyte (CFU-GM), burst-forming unit-erythroid (BFU-E) colonies]; (ii) the measure of the expression of megakaryocyte surface antigens (CD34 to CD41 and CD42); (iii) counting of proplatelets; (iv) the measurement of CYTOX activity; and (v) cell morphology (optic and electron microscopy). Oxazolidinones caused a significant decrease in BFU-E but not CFU-Mk or CFU-GM colonies. Yet, the megakaryocytic lineage was markedly affected, with a decreased differentiation of CD34+ into CD41+/CD42+ cells, an abolition of proplatelet formation and striking decrease in the numbers of large polylobulated nucleus megakaryocytes, with a complete loss of intracellular demarcation membrane system, disappearance of mitochondria, and suppression of CYTOX activity. These alterations were more marked in cells incubated with tedizolid than linezolid. These data suggest that oxazolidinones may induce thrombocytopenia by impairing megakaryocytic differentiation through mitochondrial dysfunction. Pharmacological interventions to prevent this toxicity might therefore be difficult as mitochondrial toxicity is most probably inherently linked to their antibacterial activity.

**KEYWORDS** oxazolidinone antibiotics, mitochondria, cytochrome *c*-oxidase, thrombocytopenia

Oxazolidinone antibiotics are one of the few pharmacological classes of antimicrobials endowed with a novel mode of action (namely, prevention of the formation of the initiation complex for protein synthesis) and have reached widespread clinical application over the last 20 years. Represented for longtime by linezolid only [approved in 2000 (1)], but now including also tedizolid [approved in 2014 (2)], oxazolidinones are on the World Health Organization's List of Essential Medicines as last resort options for the treatment of severe infections caused by multiresistant gram-positive organisms. Soon after its introduction in the clinics, linezolid became notorious for causing anemia and thrombocytopenia (3). The present paper focuses on thrombocytopenia, which was considered rare in registration clinical trials but is now reported to affect 30% of

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Tamara V. Milosevic and Gaëlle Vertenoeil contributed equally to this article. Author order was determined based on the fact that T. V. Milosevic initiated the close collaboration with G. Vertenoeil.

P.M.T. and F.V.B. have received speaker's and advisory boards' honoraria from Bayer and Merck (holders of marketing rights of tedizolid) and research grants from Trius Pharmaceuticals (now part of Merck) for preclinical studies of tedizolid. These companies were not involved in the design and performance of the studies presented here and did not take any part in their interpretation and/or decision to submit them to publication. The other authors have no conflict of interest to disclose in relation to the present work.

See the funding table on p. 13.

**Received** 12 April 2024
**Accepted** 4 September 2024
**Published** 19 September 2024

the patients or even more (4), and often leads to treatment discontinuation and/or repeated transfusions. This adverse drug reaction is clearly spelled out in the current labels for both linezolid and tedizolid (5, 6). Treatment over 14 days, higher dosages, and renal insufficiency (causing an elevation of the trough levels of linezolid) have been identified as significant risk factors for linezolid toxicity (6–9), suggesting concentration-dependent effects (10). The underlining molecular mechanisms remain, however, ill explored. A case report suggests an immune-mediated destruction of megakaryocytes (11), but combined pharmacokinetic, pharmacodynamic, and turnover modelling studies in humans rather point to an inhibition of the formation of platelets (10, 12, 13). In parallel, we and others have shown that oxazolidinones specifically impair mitochondrial protein synthesis [both *in vitro* and *in vivo* (14–19)], including in megakaryoblastic cell lines (15), leading to inhibition of their oxidative metabolism (16), and causing major but selective ultrastructural alterations consistent with inhibition of mitochondrial biogenesis (15–17). This has been rationalized by the existence of a high degree of homology between bacterial and mitochondrial ribosomes, with common binding sites for oxazolidinones (18–20). The link between mitochondrial dysfunction and thrombocytopenia was, however, still unclear. The present study was therefore initiated to directly assess the impact of linezolid and tedizolid on megakaryocyte maturation using the differentiation model of CD34+ cells *ex vivo*, supported by thrombopoietin (TPO) (21). We show that both oxazolidinones impair the maturation and differentiation of the megakaryocytic lineage into proplatelet-forming cells. This effect occurs in parallel with an inhibition of cytochrome *c*-oxidase activity and loss of internal structures of mitochondria in megakaryoblasts and is not reversed by increasing the TPO concentration or by adding eltrombopag (a clinically used non-peptidic TPO mimetic).

## RESULTS

### Influence of oxazolidinones on CFU-Mk, CFU-GM, and BFU-E colony formation

Figure 1 shows the effect of linezolid or tedizolid (at $C_{min}$ and $C_{max}$) on the formation of CFU-Mk-derived (megakaryocyte colony-forming units; Fig. 2) colonies (day 10) and BFU-E- (burst-forming unit-erythroid) and CFU-GM-derived (granulocyte-macrophage colony-forming units) colonies (day 13). There was no statistically significant change for CFU-Mk-derived colonies [even if analyzed separately Mk clusters containing 3 to 4, 5 to 10, or 11 to 50 cells (see Fig. S1)], suggesting that oxazolidinones did not significantly impact the overall megakaryopoiesis at this early stage of cell differentiation. There was also no statistically significant change in the number of CFU-GM-derived colonies, but these colonies were smaller. Both drugs significantly reduced the number of BFU-E-derived colonies, with tedizolid being more effective than linezolid (statistical differences observed for tedizolid at both $C_{min}$ and $C_{max}$ but only at $C_{max}$ for linezolid).

### Effect of oxazolidinones on Mk maturation

Hematopoietic stem cells express CD34 to high levels and differentiate along the Mk (megakaryocytic) lineage by progressively loosing this marker and expressing, among other markers, the CD41 glycoprotein (involved in the binding to fibrinogen and to the von Willebrand factor, and the surface receptor CD42 commonly used to discriminate between immature Mk and mature Mk (Fig. 2) (23). The impact of oxazolidinones on the relative expression of CD34, CD41, and CD42 antigens in cells of the Mk lineage was examined at day 11 of differentiation (21). We regrouped cells into three categories, representing consecutive stages of megakaryocytic differentiation: (i) those positive for CD34 (CD34+), weakly positive for CD41, and negative for CD42 (CD34+/CD41-low/CD42–); (ii) those positive for all three markers (CD34+/CD41+/CD42+); and (iii) those negative for CD34 but positive for CD41 and CD42 (CD34–/CD41+ /CD42+). These populations were quantified according to the gating method shown in Fig. S2. Samples from three independent patients were used [each run in duplicate (patient #1) or

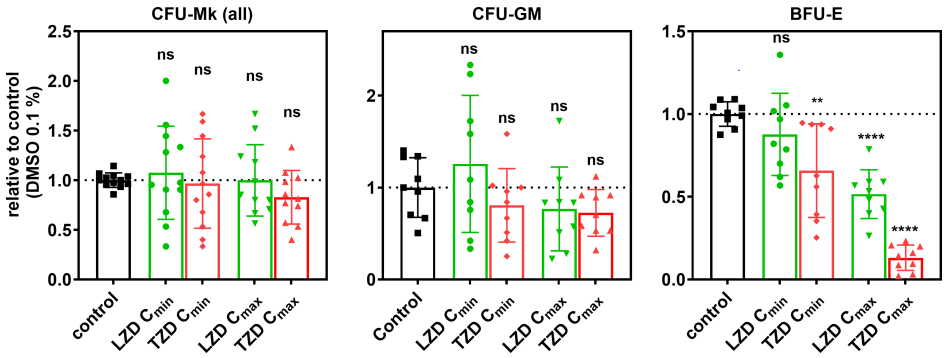


**FIG 1** Effects of oxazolidinones on the formation of colonies derived from colony-forming unit-megakaryocyte (left), colony-forming unit-granulocyte-monocyte (middle), and burst-forming unit-erythroid (right). CD34+ cells were differentiated for 10 (CFU-Mk) or 13 (CFU-GM, BFU-E) days in the presence of linezolid (LZD) and tedizolid (TZD) at concentrations corresponding to their human $C_{min}$ or $C_{max}$ total concentrations (LZD: 2.5 and 15 mg/L; TZD: 0.5 and 3 mg/L) or the vehicle [control (DMSO 0.1%)]. Colonies were counted manually in the optic microscope by two blinded investigators after CD41 labeling for CFU-Mk or for CFU-GM and BFU-E. Results are shown as the ratio of oxazolidinone-treated cells to controls [means ± SD; $n$ = 12, duplicate readings by two investigators from three samples (three patients) for CFU-Mk or $n$ = 9, triplicate readings by two investigators (one patient) and triplicate readings by one investigator (one patient) for both CFU-GM and BFU-E). For CFU-Mk, the graph shows all clusters, whether small (3–4 cells/colony), medium sized (5–10 cells/colony), or large (11–50 cells/colony), as there was no significant differences between these groups (shown in Fig. S3). Statistical analysis: one-way ANOVA with Dunnett's multiple comparison of each treatment vs control (ns, adjusted $P > 0.05$; **, adjusted $P \leq 0.01$; ****, adjusted $P \leq 0.0001$).

triplicate (patients #2 and #3)], with changes presented globally in a relative way to control in Fig. 3 (only minor and non-consistent differences were noted with the vehicle). Both linezolid and tedizolid increased, on a concentration-dependent fashion, the number of poorly differentiated cells (panel A), slightly decreased the number of intermediate-stadium cells (panel B), and markedly decreased the number of differentiated cells (panel C). Of note, tedizolid caused larger effects than linezolid, although the difference was not always significant [due to globally small changes (panel B) or important variations in response to linezolid at a low concentration (panel C)]. Thus, the data suggest that oxazolidinones, and especially tedizolid at its $C_{max}$, inhibited CD34+ differentiation into mature megakaryocytes. We looked for rescue by increasing the concentration of TPO (to 1.25 mg/L) or by adding the non-peptidic TPO receptor agonist eltrombopag (1.5 µM; 851 µg/L) but to no avail (Fig. S3).

## Influence of oxazolidinones on proplatelet formation

We examined the proplatelet formation from CD34+-derived megakaryocytes incubated with linezolid, tedizolid, or vehicle. Figure 4A shows that, at day 13, proplatelets randomly decorating cells with multiple bulges or swelling (giving them the appearance of beads connected by thin cytoplasmic strings) were clearly seen in samples exposed to the vehicle only. When incubated with linezolid or tedizolid (at their $C_{max}$), cells remained round with no pseudopods. Of note, the preparations from oxazolidinone-exposed cells contained also an abundance of small cell bodies. Cells exposed to linezolid or tedizolid at their $C_{min}$ concentrations showed some proplatelets, but they were less elongated and branched than cells exposed to the vehicle (not shown). Quantitative results are shown in Fig. 4B at day 12. Both oxazolidinones caused a sharp decrease in the proportion of Mk cells bearing proplatelets, which was obviously concentration dependent (reaching values close to zero for tedizolid at its $C_{max}$). The vehicle itself had some inhibitory effect but not statistically significant.

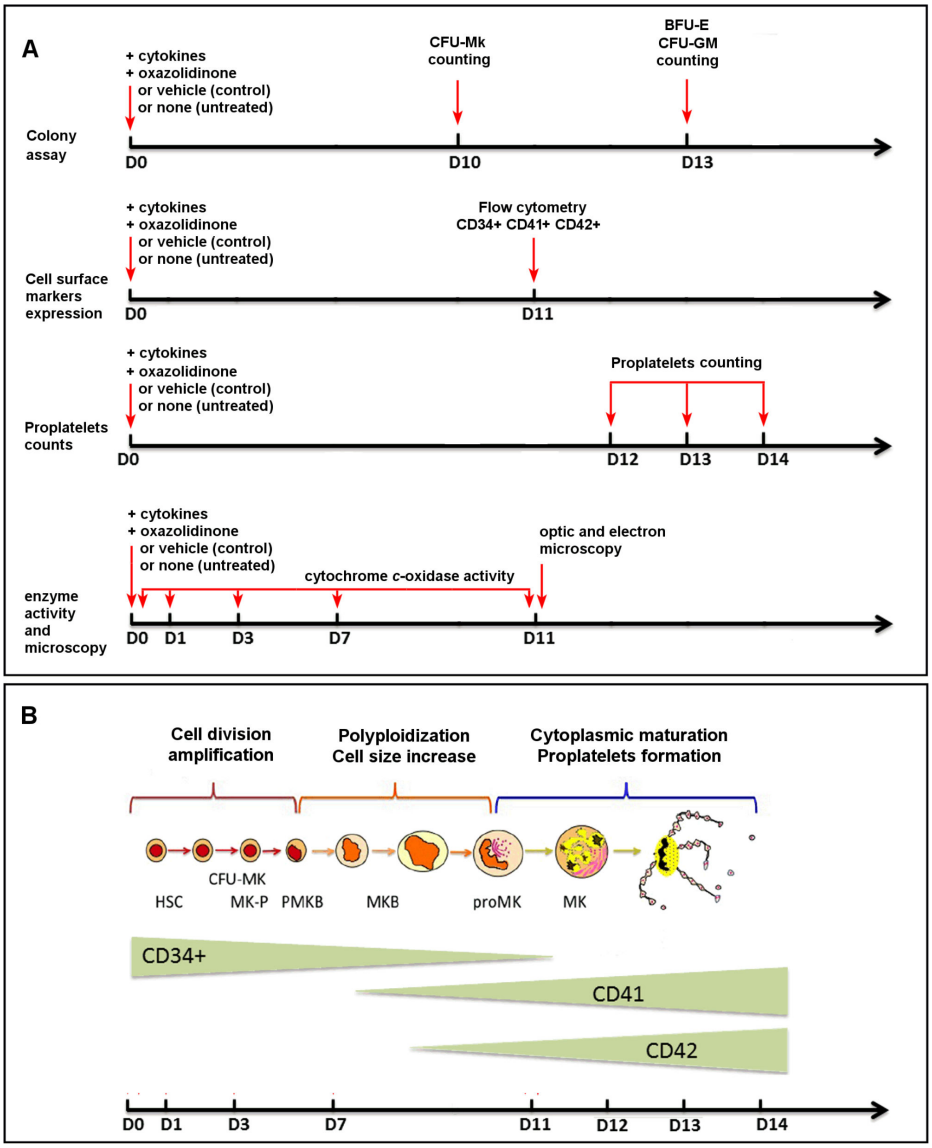


**FIG 2** Graphical representation of our protocols (A) with illustration of the differentiation process of the pluripotent hematopoietic stem cells into mature megakaryocytes and proplatelets production [(B); HSC, hematopoietic stem cells, PMKB, promegakaryoblast; MKB, megakaryoblast; Mp-K, bipotent erythroid/MK progenitor; the last stage depicts proplatelets formation; adapted from reference 22 with permission of the publisher]. The upper four lines show the days at which the different assays were performed, while the lower scheme focuses on the change in surface antigens expression. Linezolid or tedizolid was always added at day 0.

## Effect of oxazolidinones on cytochrome *c*-oxidase activity

Oxazolidinones impair mitochondrial protein synthesis and impair the activity of cytochrome *c*-oxidase (several key subunits of which are encoded by the mitochondrial DNA) in various eukaryotic cells on a time- and concentration-dependent fashion (15, 16). We, therefore, tested here whether linezolid or tedizolid would affect the activity of cytochrome *c*-oxidase in CD34+ cells during their maturation/differentiation. Figure 5 shows that both oxazolidinones caused a time- and concentration-dependent inhibition of this enzyme activity which, at day 11, reached about 50% and 80% at $C_{min}$ for linezolid and tedizolid, respectively, and, at $C_{max}$, was almost complete for linezolid and complete for tedizolid. Linezolid and tedizolid were without effect on cytochrome *c*-oxidase activity when added to the reaction mixture (16).

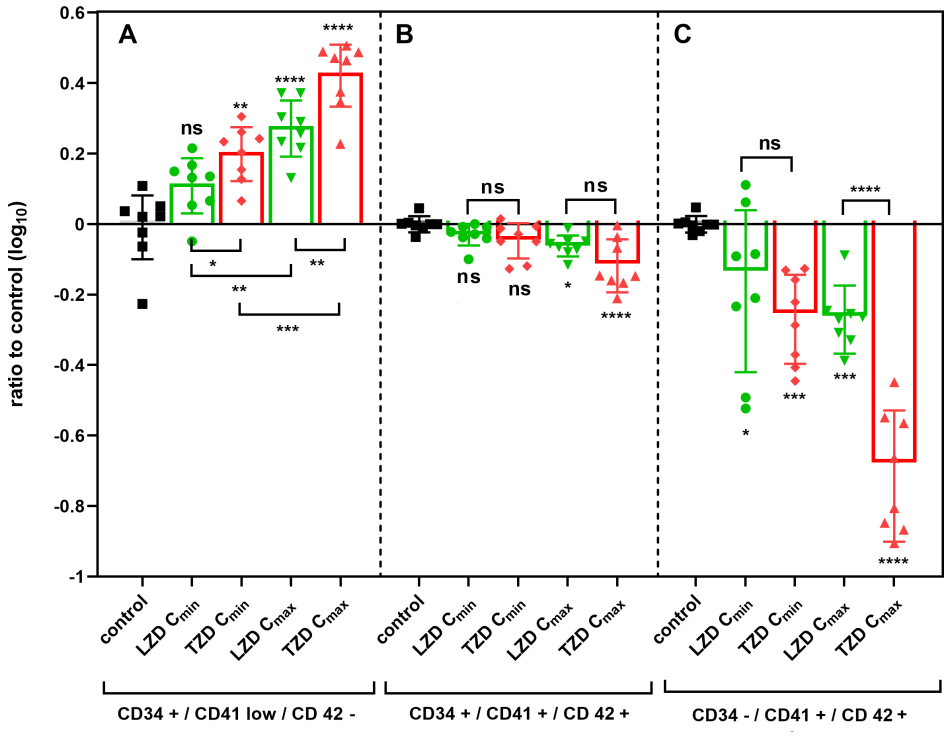


**FIG 3** Influence of oxazolidinones on megakaryocyte maturation. CD34+ cells were incubated during 11 days with linezolid (LZD) or tedizolid (TZD) at concentrations corresponding to their human $C_{min}$ or $C_{max}$ total concentrations (LZD: 2.5 and 15 mg/L; TZD: 0.5 and 3 mg/L) or with their vehicle (DMSO 0.1%; control), stained with specific labeled antibodies (anti-CD34-PE, anti-CD41-FITC, or anti-CD42-APC) at 4°C, and analyzed by flow cytometry. The data are shown as variation over control (logarithmic scale). (A), total counts of CD34 positive, CD41 weakly positive, and CD42 negative cells (CD34+/CD41 low/CD42−); (B), total counts of CD34, CD41, and CD42 positive cells (CD34+/CD41+/CD42+); (C) total counts of CD34 negative, CD41 and CD42 positive cells (CD34−/CD41+/CD42+). The ordinate ($log_{10}$ scale) shows the ratio of oxazolidinone-treated cells to control for each of these three groups ± SD [$n$ = 8; triplicate readings from two samples (two patients) and duplicate readings from an additional sample (one patient)]. Statistical analysis: (i) comparisons of oxazolidinone-treated cells vs the corresponding control—one-way ANOVA with Dunnett's multiple comparison of each treatment vs control [adjusted $P$ value symbols are shown above (A) or below (B and C) each column]; (ii) effect of the concentration of LZD or TZD on the change in (CD34+/CD41 low/CD42−) cells (A): one way ANOVA; (iii) comparison between oxazolidinones at equitherapeutic concentrations (LZD vs TZD at their $C_{min}$ or its $C_{max}$): unpaired two-tailed $t$-test [$P$ value symbols are shown below (A) or above the compared columns (B and C)]; symbols: ns, $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$, ****, $P \leq 0.0001$.

## Morphological characteristics of megakaryocytes in control conditions and after incubation with oxazolidinones

CD34+ cells exposed for 11 days or to linezolid or tedizolid (at $C_{max}$) or to their vehicle (DMSO 0.1%) were also examined in optic and electron microscopy.

In optic microscopy (Fig. S4), cells exposed to the vehicle only were often large, with multiple nuclear sections (polylobulated nuclei), occasional dense bodies inside or budging from cells, and, more rarely, intracellular small, dense granules. In linezolid- or tedizolid-treated samples, large cells with multiple nuclear sections were much less frequent, leaving multiple large immature cells (with no or little evidence of polylobulation) as well as numerous dense bodies, some of them seeming extracellular, although often adjacent to cells. Cells containing small dense granules were also much more frequent, and cells with large, apparently empty vacuoles were also occasionally seen.

In the electron microscope (Fig. 6), cells exposed to the vehicle only had often a large cytoplasm with a well-developed demarcation membrane system (DMS), numerous α-granules, and large mitochondria with well-developed, characteristic cristae. In contrast, the DMS and typical mitochondria were almost completely absent in

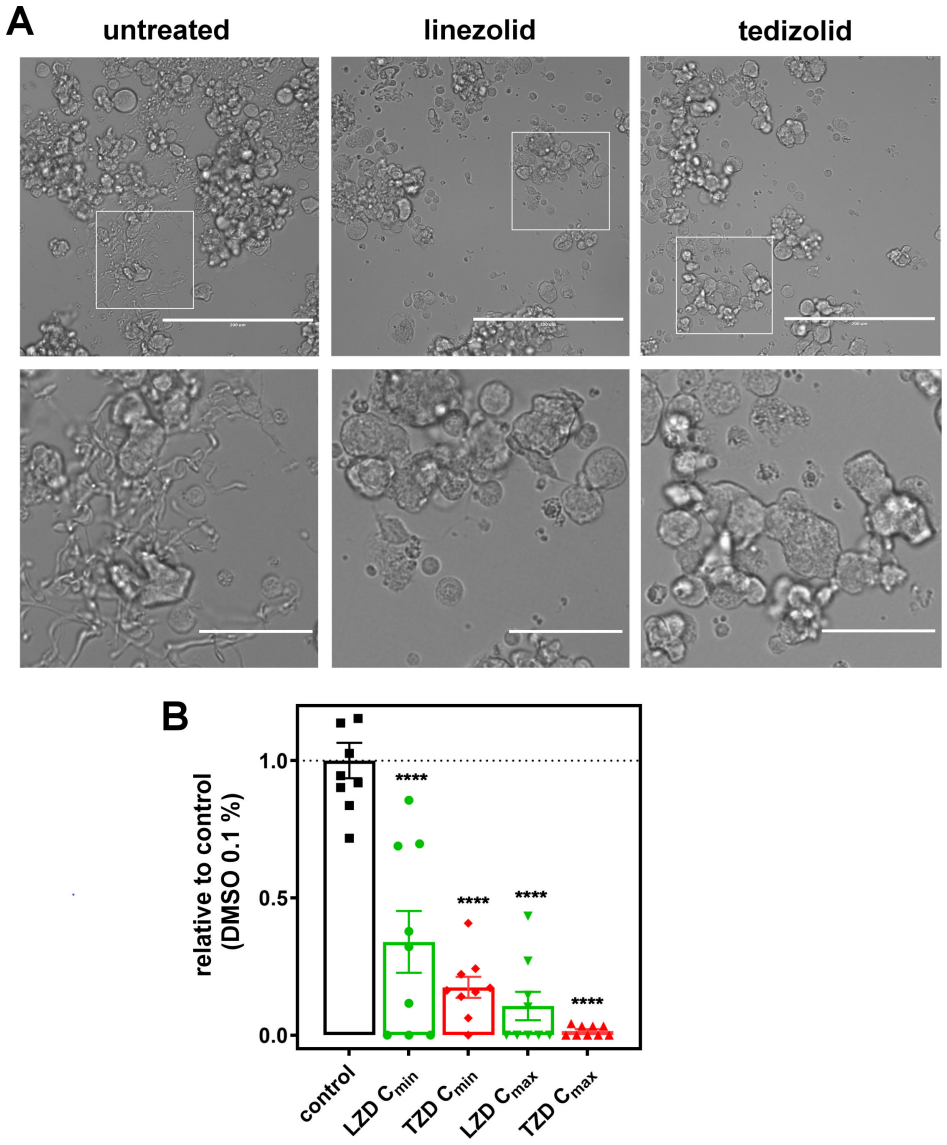


**FIG 4** Influence of oxazolidinones on the proplatelet (PP) formation process. CD34+ cells were subjected to differentiation without further treatment or in the presence of linezolid (LZD) or tedizolid (TZD), or of the vehicle (DMSO 1%; control) and examined for differentiation and formation of proplatelets by optic microscopy. (A) Morphological appearance (representative images) at day 13 of differentiation for untreated cells or for cells incubated in the presence of linezolid or tedizolid (at a concentration corresponding to their total human $C_{max}$ (15 and 3 mg/L, respectively); the upper row shows a general view of the preparations (scale bar: 200 µm) and the lower row a close-up view (the corresponding zone is highlighted by a white square on the top row; scale bar: 50 µm), showing an abundance of proplatelets surrounding untreated cells, whereas these are absent from cell exposed to linezolid or tedizolid, note also the abundance of small bodies in these samples. (B) Quantitative evaluation at day 12 of differentiation based on the enumeration of Mks showing at least one proplatelet extension as determined by counting at least 100 Mks in each replicate of each experimental group. Results are shown as the ratio of oxazolidinone-treated cells to controls (DMSO 0.1%). Antibiotic-treated cells were exposed to linezolid or tedizolid at concentrations corresponding to their human $C_{min}$ or $C_{max}$ total concentrations (LZD: 2.5 and 15 mg/L; TZD: 0.5 and 3 mg/L). The results are expressed as the mean ± SD of triplicate readings of samples from three patients. Statistical analysis: one-way ANOVA with Dunnett's multiple comparison of each treatment vs vehicle only (ns, adjusted $P > 0.05$; ***, adjusted $P \leq 0.01$; ****, $P \leq 0.0001$).

linezolid- or tedizolid-treated samples, while cells contained (i) numerous large vacuoles with an electron-lucent content straddled by reticular but often disorganized

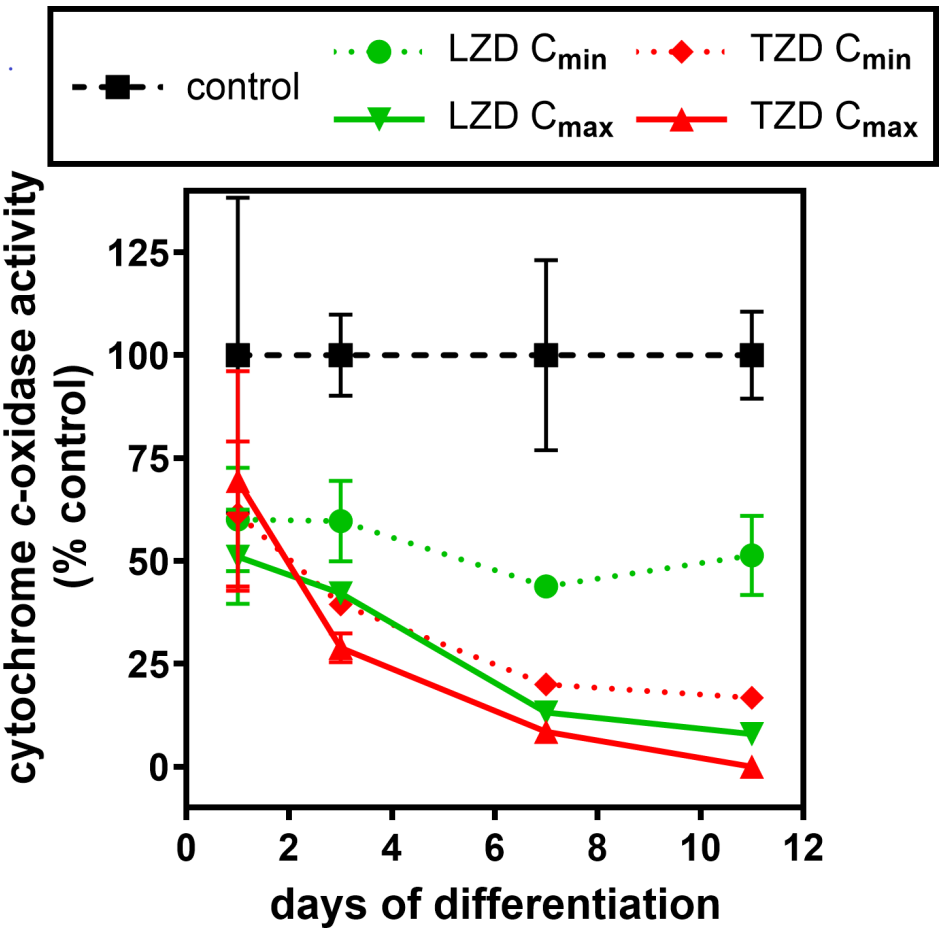


**FIG 5** Influence of oxazolidinones on the activity of cytochrome *c*-oxidase of CD34+ cells during differentiation. CD34+ cells were incubated for up to 11 days with the vehicle [control (DMSO 0.1%)] or with linezolid (LZD) or tedizolid (TZD) at concentrations corresponding to their human $C_{min}$ or $C_{max}$ total concentrations (LZD: 2.5 and 15 mg/L; TZD: 0.5 and 3 mg/L), and collected for measurement of cytochrome *c*-oxidase activity. The ordinate shows the activity of the enzyme normalized to each sample cell protein content and expressed in percentage from corresponding control [vehicle only (DMSO 0.1%)]. Data are the mean ± SD (when not visible, the SD bar is smaller than the symbol) of triplicate values (cells from one patient). Statistical analysis: two-way ANOVA with Tukey's multiple comparisons test—no significant difference was found after 1 day of differentiation between control and oxazolidinone-treated cells, but from day 3, all oxazolidinone-treated cells showed a significant difference compared to controls ($P < 0.05$). Tedizolid also caused a statistically significantly larger decrease of cytochrome *c*-oxidase activity than linezolid for most of the values recorded at 3, 7, and 11 when compared at their respective $C_{min}$ or $C_{max}$ values.

moderately electron-dense structures and occasional small dense bodies (possibly swollen mitochondria) and (ii) granules of about 0.25 µm diameter with a moderately electron-dense granular material (also visible but less frequently in controls). Apparently, damaged cells with large highly electron dense bodies, which could be the hallmark of ongoing apoptotic processes, were also frequent after treatment with linezolid or tedizolid. While only few linezolid- or tedizolid-treated cells still showed α-granules, most cells contained an abundance of intracellular membranes bearing ribosomes (endoplasmic reticulum), suggesting unimpaired synthesis for proteins encoded by the nuclear genome.

## DISCUSSION

While linezolid hematological toxicity has been described in patients since its early clinical approval (3), the present study represents, to the best of our knowledge, one

of the first direct assessments of the ability of two drugs of this class to impair *ex vivo* the process of differentiation of megakaryocytic lineages from hematopoietic stem cell/progenitors. Our key observation is that oxazolidinones specifically hinder the differentiation of the megakaryocytic lineage at therapeutically pertinent concentrations, in parallel with an inhibition of cytochrome *c*-oxidase activity in mitochondria and the appearance of ultrastructural alterations of these organelles. These alterations could possibly constitute a cellular basis for the thrombocytopenia observed in treated patients.

We show that oxazolidinones markedly reduce the maturation of promegakaryoblasts and megakaryoblasts into fully differentiated proplatelet-producing megakaryocytes *ex vivo* at drug concentrations that are only slightly higher than their minimal inhibitory concentrations against susceptible bacteria (24) but which are pertinent of the human therapeutic use of these antibiotics since we used concentrations mimicking their respective human $C_{min}$ and $C_{max}$ (see reference 16 for further discussion). This illustrates the intrinsically narrow therapeutic margin of these drugs and explains why thrombocytopenia is more frequent when trough concentrations of linezolid become elevated, increasing the overall drug exposure (8, 25). *In vivo*, the entire process of platelet release spans approximately 14 days, which explains why platelet loss becomes noticeable only after this duration of treatment (26), even though immediate effects, possibly related to the distinct immunological mechanism(s), may also occur (11, 12).

Antiproliferative and cytotoxic effects of oxazolidinones have been described in cultured cells (20) but usually at much higher concentrations than those used here. Thus, under these clinically relevant conditions of exposure, the megakaryocytic progenitors themselves remain unaffected, as evidenced by the absence of any decrease in CFU-Mk numbers, while a reduced abundance in BFU-E colonies is noticed. The specificity of this anti-proliferative or cytotoxic effect for the erythroid lineage could be related to the facts that (i) BFU-E colonies require longer time for maturation than CFU-Mk precursors, (ii) mitochondria are also involved in several stages of erythroid differentiation, but (iii) precursors of red blood cells lose their mitochondria along the maturation process (27). We did not investigate further these hypotheses, as the focus of this work was on the megakaryocytic lineage, but this may explain why anemia in also commonly observed in linezolid-treated patients (5). Noteworthy, however, a concentration effect is likely because the reduction in BFU-E was only observed in cells exposed to concentrations mimicking $C_{max}$. Lastly, the formation of granulocytic-monocytic cells was not affected, in-line with the observation that neutropenia is only rarely reported in linezolid-treated patients (7, 26).

Limiting the discussion to thrombocytopenia, platelet formation and release involve a complex process of differentiation from hematopoietic stem and progenitor cells to megakaryocytes. These cells undergo endomitosis and maturation to form long cytoplasmic extensions (proplatelets) that will eventually allow platelets release through fission (28). Mitochondria have been clearly involved in megakaryopoiesis and platelet release (29) since high energy production is needed for endomitosis and proplatelet production (30). A critical role of reactive oxygen species for triggering megakaryocyte proliferation, differentiation, maturation, and platelet activation has been advocated (30, 31), with evidence for their mitochondrial origin (32). This is in-line with our observations that the impairment of megakaryocyte maturation and proplatelet formation is accompanied by an inhibition of the activity of cytochrome *c*-oxidase and major structural alterations of mitochondria. Previous studies in other cell types showed that oxazolidinones are mitotoxic (33), specifically inhibit the synthesis of proteins encoded by the mitochondrial genome (16, 20) [while cytoplasmic protein synthesis remains unimpaired (19, 20)], and induce a marked impairment of the mitochondrial oxidative metabolism (16). Conversely, early work with eperezolid [an oxazolidinone structurally similar to linezolid but causes a more severe thrombocytopenia, leading to the discontinuation of its development (34)] showed that this oxazolidinone did not inhibit the growth of so-called "rho-zero" cells, lacking mitochondrial DNA, but markedly

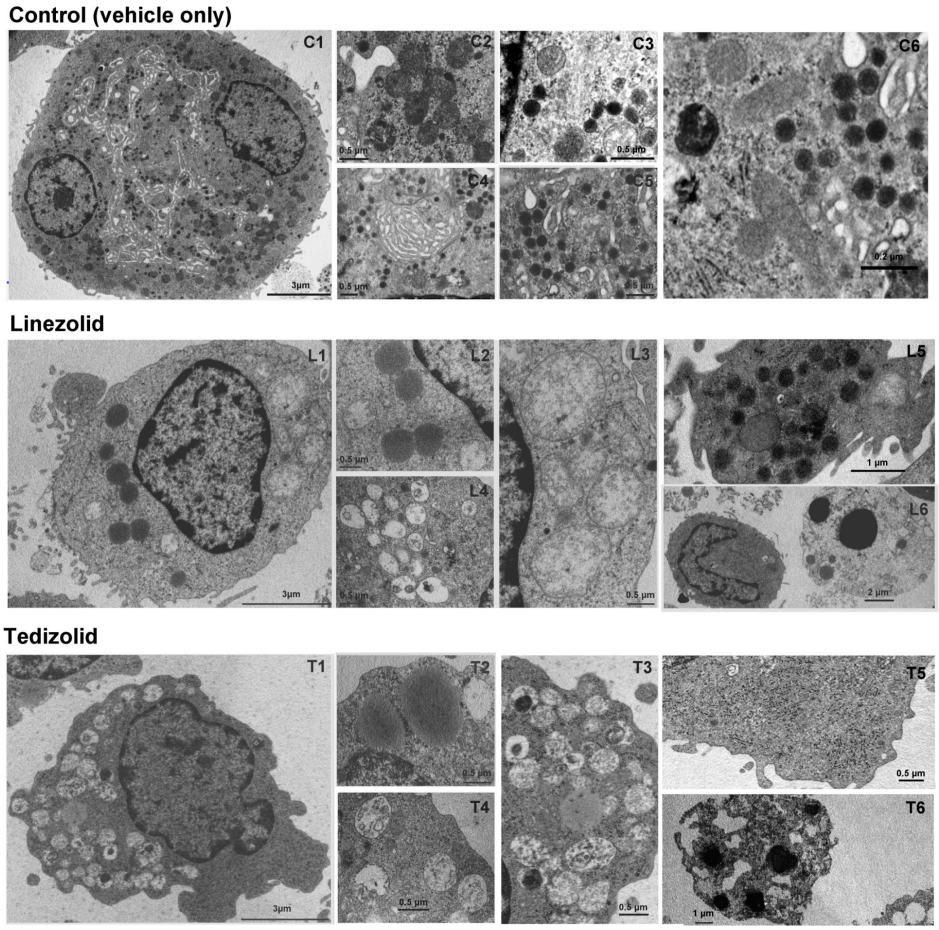


**FIG 6** Electron microscopy of CD34+-derived megakaryocytes cultured in control conditions (vehicle only) or with oxazolidinones. Cells were exposed to the vehicle only (DMSO 0.1%; control) or to linezolid or tedizolid [at a concentration corresponding to their total human $C_{max}$ (15 and 3 mg/L, respectively)] for 11 days. Sections were cut at 60 nm thickness and stained with uranyl acetate and lead citrate. C1–C6, control cells; C1, general view of a control cell showing numerous α-granules, well-developed mitochondria, with the entire cytoplasm being dissected into different sized territories by the DMS; C2–C6: close-up views showing details of mitochondria, α-granules, and demarcation membranes. L1–L7, linezolid-treated cells; L1, general appearance of a progenitor cell illustrating the presence of bodies with a dense, granular content and swollen mitochondria; L2–L4, close-up views showing more details of the dense granules and large, largely empty vacuoles with occasional small dense bodies; L5, cluster of α-granules surrounding a body with dense granular content; L6, intact cell with a curved nucleus with an adjacent damaged cell with large dense intracellular bodies. T1–T7, tedizolid-treated cells; T1, general appearance of a progenitor cell showing an abundance of large, partially empty vacuoles; T2, dense bodies with granular content; T3, empty vacuoles with occasional dense bodies surrounding a dense body with granular content; T4, empty vacuoles with residual material; T5, cytoplasm with abundance of membrane-bound ribosomes (endoplasmic reticulum); T6, damaged cell with conspicuous large intracellular dense bodies.

impaired that of the parent cells containing mitochondrial DNA (20). Taken together, these observations indicate that mitochondria are the main, if not exclusive, targets of oxazolidinone toxicity.

Oxazolidinone effects on mitochondria can somehow be compared with the Pearson's syndrome, in which rearrangements of the mitochondrial genome between direct DNA repeats lead to a multisystem mitochondrial disorder that includes anemia and thrombocytopenia (35). These patients show a high lactate/pyruvate molar ratio leading to lactic acidosis. Intriguingly, lactic acidosis is a rare but threatening event also observed in patients exposed to linezolid (5, 36). Electron micrograph of lymphocyte mitochondria from a patient with Pearson's syndrome also revealed a swelling of mitochondria that

bears much similarity to what we described for mitochondria of HL-60 promyelocytes exposed to linezolid or tedizolid (16).

In a translational perspective, we also show that the toxic effects of oxazolidinones are not reversed by increasing TPO concentration or by adding the non-peptidic TPO-receptor agonist eltrombopag. This suggests a complete blockade of a key step in the signaling pathway, consistent with the images of disorganized mitochondria we observed here. Thrombopoietin (via JAK2/pSTAT5/pSTAT3 signaling) stimulates hematopoietic multipotent and progenitor cells (or a subset of them), rapidly upregulating their mitochondrial activity to trigger their exit from dormancy and differentiation into megakaryocytes (32). It would therefore be useful to determine at which time point in the maturation of megakaryoblasts and differentiation process of megakaryocytes, oxazolidinones are acting, as this may better guide the development of protective strategies using TPO or one of its non-peptidic TPO-receptor agonists.

In our experiments, tedizolid appears as more potent than linezolid to inhibit megakaryoblasts differentiation and mitochondrial functions. As previously discussed (16), this probably results from its stronger interaction with ribosomes, explaining its higher intrinsic activity on bacteria (37, 38). This is also probably the case vs mitochondrial ribosomes, but this specific point has, however, not been experimentally investigated. In the clinics, this higher intrinsic toxicity could be mitigated by a once-daily schedule of administration, which would allow for more prolonged recovery periods, and by a shorter treatment duration, which would limit the global exposure to the drug (14). Safety data in clinical practice are limited with tedizolid and often restricted to small sample size, preventing the detection of rare adverse events (see, e.g., references 39–41). Yet, a recent analysis of FDA pharmacovigilance data reported a similar proportion of adverse events, including thrombocytopenia, for both clinically available oxazolidinones (42). This contrasts with registration trials, which suggested an improved safety profile for tedizolid over linezolid (43). This discrepancy could be ascribed to the large post-marketing off-label use of tedizolid, which includes indications requiring a long treatment duration, such as mycobacterial infections and osteomyelitis/joint infections (42).

Our study has some limitations. First, we used cells from patients with hemochromatosis and not from healthy individuals. This was necessary to obtain a sufficiently high number of cells, as differentiation of the megakaryocytes from CD34+ cells has a low yield *ex vivo*. But an untreated control was included in each experiment, showing that these cells behave as expected. Second, we did not study the ploidy status of the cells during maturation as our focus was on mitochondria, based on the known mechanism of action of oxazolidinones. This could be the topic of future investigations. Third, cells were exposed to constant concentrations of oxazolidinones, which do not mimic the fluctuations observed over time in treated patients. This prevented us from seeing the recovery capacity of the cells (16), which is difficult to study i*n vitro*, especially for a process developing over several days of cultures, as it is the case for cell differentiation.

In summary, and in spite of these limitations, we provide evidence for a major and largely specific effect of oxazolidinones on the differentiation and maturation process of the megakaryocytic lineage from human hematopoietic stem cells exposed to clinically relevant concentrations. Mitochondria seem to emerge as a primary target not only because these organelles serve as targets for these antibiotics, as in many other tissues, but also because they play a crucial signal-transmitting role for thrombopoietin, the natural stimulating factor for the production of platelets via megakaryocytic differentiation and maturation. While these observations may explain many aspects of the hematological toxicity of oxazolidinones, the impact of protective or correcting measures, beyond controlling serum levels and treatment duration, might remain limited because the destructive effect of the antibiotics on the mitochondria is intrinsically linked to their antibacterial activity.

## MATERIALS AND METHODS

### Drugs and cytokines

Linezolid and tedizolid were obtained as microbiological standards from Rib-X Pharmaceuticals (presently Melinta Therapeutics, New Haven, CT), and Trius Pharmaceuticals (San Diego, CA), now part of Merck & Co. (Kenilworth, NJ). They were prepared in DMSO [final concentration 0.1% (vehicle; no difference observed when comparing cells in culture medium only or in culture medium added by 0.1% DMSO for all measured parameters; see Fig. 2 for definition of controls)] (16) and used at concentrations corresponding to their average total maximal ($C_{max}$) and minimal ($C_{min}$) concentrations typically observed in humans receiving conventional therapy [15 and 2.5 mg/L, and 3 and 0.5 mg/L for linezolid (5) and tedizolid (6), respectively]. Cytokines were purchased from Miltenyi Biotec GmbH [TPO, IL-6, G-CSF (granulocyte-colony-stimulating factor); Bergisch Gladbach, Germany], STEMCELL Technologies Inc. [IL-3, SCF (stem cell factor)]; Vancouver, BC], or Janssen-Cilag [EPO (erythropoietin); Beerse, Belgium]. Eltrombopag (olamine) was a research sample from Novartis (Basel, Switzerland).

### Isolation of CD34+ cells and general culture conditions

CD34+ cells were prepared from leftover blood samples of patients with hemochromatosis, with the approval of the local ethical committee. The reason for the selection of these patients as source of cells as well as the isolation method is described in the supplemental methods. Unless stated otherwise, cells were cultured in StemSpan SFEM medium (serum-free medium for culture and expansion of hematopoietic cells; StemCell Technologies) and stimulated for differentiation with TPO (10 ng/mL) and SCF (5 ng/mL).

### General experimental design

CD34+ cells were allowed to differentiate *ex vivo* into erythroid, granulocytic, or megakaryocytic cells over a period of up to 14 days by exposure to the appropriate cytokines without further addition (untreated cells), or with addition of linezolid or tedizolid (antibiotic-treated cells) or their vehicle (DMSO 0.1%; controls). Colony assays were performed at day 10 (CFU-Mk) or day 13 (BFU-E and CFU-GM). Cytochrome *c*-oxidase activity was measured on whole-cell homogenates at days 0, 1, 3, 7, and 11. The relative expression of cell surface markers (CD34, CD41, and CD42) was assessed at day 11. Counts of proplatelets were performed at days 12, 13, and 14. Figure 2 shows a graphical representation of the timeline of these determinations along with the differentiation process of hematopoietic stem and progenitor cells (promegakaryoblasts; CD34+) into megakaryocytes and proplatelet-producing cells (CD41+ and CD42+ cells).

### Colony-forming unit assays

We followed the procedures set forth by StemCell Technologies (44, 45) and summarized in the supplemental material. In brief, for CFU-Mk assays, 1,000 cells per slide were cultivated in MegaCult-C medium with lipids and collagen (gelling agent) in the presence of TPO (10 ng/mL) and SCF (25 ng/mL). CFU-Mk readings were made on fixed [methanol:acetone (vol:vol 1:3)] samples stained with MegaCult-C Staining Kit for CFU-Mk (StemCell Technologies). For BFU-E and CFU-GM assays, 1,100 CD34+ cells (dilution of cells in Iscove's Modified Dulbecco's Medium supplemented with 2% fetal bovine serum) were plated per dish in MethoCult methylcellulose-based medium containing IL-3 (10 ng/mL), EPO (1 U/mL), SCF (25 ng/mL), G-CSF (20 ng/mL), and IL-6 (10 ng/mL). Readings were made using optic microscopy. For all assays, colonies were counted independently by two evaluators unaware of the treatment of the samples examined.

### Measurement of cytochrome *c*-oxidase activity

The activity of this mitochondrial enzyme was measured and activity calculated as previously described, taking into account the protein content of each sample (16).

### Expression of Mk cell surface antigens (flow cytometry)

Harvested and PBS-washed cells were incubated with human IgG (10 min; 4°C), brought to a density of $10^6$ cells/100 µL in PBS-EDTA-FBS, and incubated with phycoerythrin-conjugated anti-CD34 (#130-081-002, Miltenyi Biotec), fluorescein isothiocyanate-conjugated anti-CD41 (#130-105-559, Miltenyi Biotec), and allophycocyanin-conjugated anti-CD42 (#303912, BioLegend, San Diego, CA) murine monoclonal antibodies (dilution: 1/40; 30 min: 4°C), and washed (PBS-EDTA-FBS) and re-suspended in the same solution. Flow cytometry was made with a BD FACS Calibur instrument (BD Bioscience, Franklin Lakes, NJ) with CellQuest software and analyzed using FlowJo software.

### Proplatelet formation assay

Proplatelet formation was determined by counting megakaryocytes showing at least one proplatelet extension, using an EVOS XL digital inverted microscope with 20× magnification (Thermo Fisher Scientific, Waltham, MA), with readings made in duplicate by two independent observers unaware of the treatment of the samples.

### Optic and electron microscopy

Control and oxazolidinone-treated cells (11 days of differentiation) were collected by low-speed centrifugation (200 × *g*; 5 min) in conically shaped miniature tubes and fixed with glutaraldehyde and osmic acid as previously described (16), dehydrated by successive immersion (with 10 min soaking each time) in 30%, 50%, 70%, 85%, and 100% ethanol, and embedded in Eponate 12 Kit resin (#18012; Ted Pella Inc., Redding, CA). Semi-thin sections (1 µm; toluidine blue staining) were used for optic microscopy and ultrathin sections (60 nm; uranyl acetate/lead citrate staining) for electron microscopy (Philips CM-12, Philips, Amsterdam, The Netherlands).

### Statistical analyses

GraphPad Prism version 10.2.1 (GraphPad Software, Inc., San Diego, CA) was used.

## ACKNOWLEDGMENTS

We thank Prof. Carine Michiels (Université de Namur, Namur, Belgium) for help in the preparation of samples for electron microscopy and Novartis Pharma AG, Basel, Switzerland, for providing us the sample of eltrombopag used in our studies. Virginie Mohymont and Katia Santos-Saial (Université catholique de Louvain), and Lidvine Genet and Caroline De Bona (Université de Namur) provided dedicated technical assistance. We thank Dr. Nicolas Dauguet for flow cytometry assistance.

T.V.M. was supported by the Université catholique de Louvain and G.V. by a PhD Mandat Aspirant of the FRS-FNRS Belgium. F.V.B. is Research Director of FRS-FNRS Belgium. This work was supported by the general budget awarded from various sources to F.V.B. Funding to S.N.C. is acknowledged from Ludwig Institute for Cancer Research, Fondation contre le cancer, Salus Sanguinis, and Fondation "Les avions de Sébastien", projects Actions de recherche concertée (ARC) 16/21–073 and Wel Research Institute WelBio F 44/8/5 - MCF/UIG – 10955. Funding of W.V. is acknowledged from Ligue Nationale Contre le Cancer ("Equipe labellisée 2016", H.R.), Institut National du Cancer (INCA-PLBIO-2015, I.P.), and Institut National de la Santé et de la Recherche Médicale (Inserm).

T.V.M. and G.V. designed and performed all experiments and analyzed the results. W.V. examined and interpreted the electron microscopy images presented here and made substantial contributions to the interpretation of all data. S.N.C. provided guidance

throughout the study and made essential contributions in the interpretation of all data. P.M.T. and F.V.B. initiated the work, supervised all steps of the studies, and actively participated in the interpretation of all data. T.M., P.M.T., and F.V.B. wrote the paper. All authors commented, made corrections, and approved the submitted version.

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## FUNDING


| Funder | Grant(s) | Author(s) |
|---|---|---|
| Ludwig Institute for Cancer Research | | Stefan N. Constantinescu |
| Stichting Tegen Kanker (Fondation Contre le Cancer) | | Stefan N. Constantinescu |
| Salus Sanguinis | | Stefan N. Constantinescu |
| Les avions de Sébastien | | Stefan N. Constantinescu |
| Actions de recherche Concertée | 16/21-073 | Stefan N. Constantinescu |
| Wel research Institute Welbio | F 44/8/5 - MCF/UIG - 10955 | Stefan N. Constantinescu |
| Ligue Contre le Cancer (French League Against Cancer) | | William Vainchenker |
| Institut National Du Cancer (INCa) | INCA-PLBIO-2015 | William Vainchenker |
| Institut National de la Santé et de la Recherche Médicale (Inserm) | | William Vainchenker |


## DATA AVAILABILITY

All data are available from the corresponding author upon request.

## ETHICS APPROVAL

Collection of white blood cells from patients with hemochromatosis for research purposes was approved by the commission d'éthique hospitalo-facultaire from the cliniques universitaires Saint-Luc and Université catholique de Louvain (reference: 2021/12 AOUT/343).

## ADDITIONAL FILES

The following material is available online.

### Supplemental Material

**Supplemental material (AAC00533-24-s0001.pdf).** Supplemental methods; Figures S1 to S4.

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