Myelosuppression in Patients Treated With the Telomerase Inhibitor Imetelstat Is Not Mediated Through Activation of Toll-Like Receptors

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INTRODUCTION

- Two recent *New England Journal of Medicine* publications reported that imetelstat (GRN163L) is active in patients with essential thrombocythemia (ET)[1] or primary, post-ET, and post-polycythemia vera myelofibrosis.[2]
- Imetelstat (GRN163L) is a covalently lipidated 13-mer N3′-P5′ thio-phosphoramidate (NPS) oligonucleotide (**Figure 1**) that is complementary to the RNA template region (hTR) of the telomerase enzyme.[3]
- Imetelstat has a covalently bound 5′ lipophilic (palmitoyl) group to increase cell permeability and tissue distribution.[3]
- Imetelstat has long tissue residence time in bone marrow, spleen, and liver (0.19–0.51 µM observed in human bone marrow at 41–45 hours post 7.5 mg/kg dose).[4]
- Imetelstat is a potent direct competitive inhibitor of telomerase reverse transcriptase enzyme activity (half maximal inhibitory concentration [IC_{50}] = 0.5-10.0 nM [cell-free]) (**Figure 2**).[3,6,7]
  - Inhibits telomerase activity *in vitro* across multiple cancer cell lines.[3,7]
  - Induces reduction in telomere length; by inhibiting the RNA component of telomerase, hTERT is reduced and telomere length decreases (**Figure 2**).[7,8]
- Imetelstat does not elicit its effect through an antisense inhibition of protein translation.

Imetelstat 13-mer[1]
5′-TAGGGTTAGACAA-3′

Mipomersin 20-mer[a,†]
5′-G*-C*-C*-U*-C*-dA-dG-dT-dC-dT-dG-dmC-dT-dT-dmC-0′-C*-A*-C*-C*-3′
(d = 2′-deoxy, * = 2′-O-(2-methoxyethyl))

Genasense Bcl-2 inhibitor 18-mer[a,‡]
5′-TCTCCCAGCGTGCGCCAT-3′

ISIS 22783 Bcl-xL inhibitor 20-mer[a,‡]
5′-CTGGATCCAAGGCTCTAGGT-3′

[a]Approved cholesterol-reducing agent. Asterisks in nucleotide sequence denote 2′-O-(2-methoxyethyl) nucleosides.
[‡]Investigational cancer therapies.

Figure 1. Imetelstat sequence is short, unlike antisense agents

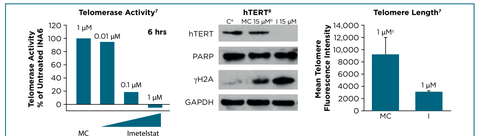

Figure 2. Imetelstat is a direct competitive inhibitor of telomerase, with downstream reductions in hTERT, and telomere length

C, control; I, imetelstat; MC, mismatch control; hTERT, human telomerase reverse transcriptase; PARP, poly ADP ribose polymerase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase. [a]Untreated control; [b]MM1 mismatch; [c]57S mismatch.

Figures included with permission.[7,8]

- Toll-like receptors (TLRs) are proteins with domains that recognize pathogen-associated molecular patterns and stimulate downstream signaling to trigger innate immune responses[9,10] (**Figure 3**).

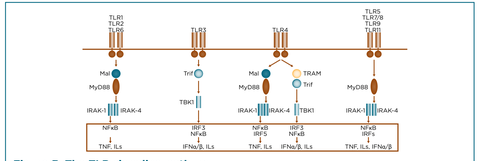

Figure 3. The TLR signaling pathway

Figure included with permission.[10]

- Synthetic, single-stranded DNA with CpG (unmethylated deoxycytidyldeoxyguanosine) dinucleotide motifs, characteristic of bacteria and virus genomes, activate the innate immune response through TLR9 signaling (**Figures 3 and 4**).[11]
  - Minimal oligonucleotides that activate human TLR9 comprise 2 CpG islands separated by 6 to 10 nucleotides, where the first CpG motif is preceded by the 5′-thymidine and the elongated poly-thymidine tail at the 3′ end of the oligonucleotide.
  - Oligonucleotides shorter than 21 nucleotides are less likely to activate TLR9.
- Phosphorothioate oligonucleotides with the above properties bind to and activate TLR9.[12]
- Treatment with imetelstat has been associated with thrombocytopenia,[1,2] and it was recently proposed that the thrombocytopenia observed in patients with myeloproliferative neoplasms (MPN) treated with imetelstat may occur through off-target effects, with the hypothesis that imetelstat acts as a ligand for TLRs such as TLR9.[12]
  - An association between TLR activation and lipopolysaccharide-induced thrombocytopenia has been demonstrated.[13]
- However, the sequence of imetelstat is shorter than the minimal sequence to activate TLR9, and it lacks CpG motifs spaced by 6 to 10 nucleotides required for activation (**Figure 4**).

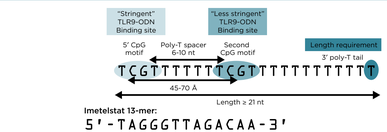

Figure 4. Features required for TLR9 activation compared with imetelstat sequence

Figure included with permission.[11]

OBJECTIVE

- To assess whether imetelstat activates TLRs.

MATERIALS AND METHODS

NFκB-Inducible SEAP Reporter Assay for TLR Activity

- HEK293 cell lines stably co-expressing a human TLR gene (TLR2, -3, -4, -5, -7, -8, or -9) and an NFκB-inducible SEAP (secreted embryonic alkaline phosphatase) reporter gene were used to test whether experimental agents activate TLR signaling (**Figure 5**).
- **Positive control ligands for TLR activation**
  - hTLR2: heat-killed *Lysteria monocytogenes* at 10^8 cells/mL
  - hTLR3: Poly(I:C) HMW, 1 µg/mL
  - hTLR4: *Escherichia coli* K12 LPS, 100 ng/mL
  - hTLR5: *Salmonella typhimurium* flagellin, 100 ng/mL
  - hTLR7: Imiquimod, 1 µg/mL
  - hTLR8: CL075, 1 µg/mL
  - hTLR9: CpG ODN 2006 at 100 ng/mL
- **Experimental agents**[a]
  - Imetelstat: 5′-R-TAGGGTTAGACAA-NH2-3′
  - Mismatch oligo: 5′-R-TAGGTGTAAGCAA-NH2-3′
  - Sense oligo: 5′-AACAGATTGGGAT-R-3′

[a]*R in oligonucleotide sequences refers to the covalently bound lipophilic (palmitoyl) group of the molecules.*

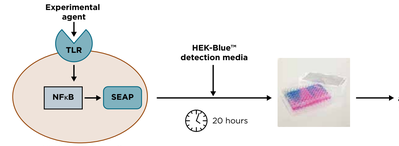

Figure 5. NFκB-inducible SEAP reporter assay for TLR activity

RESULTS

TLR Activity with Imetelstat and Positive Controls

- Treatment with clinically relevant concentrations of imetelstat had no stimulatory effect on TLR2, TLR3, TLR4, TLR5, TLR7, or TLR9 (**Figure 6**).
- Treatment with imetelstat caused induction of TLR8 that was higher than that of the untreated control, but the observed increase was substantially lower than in the positive control (**Figure 6C**).

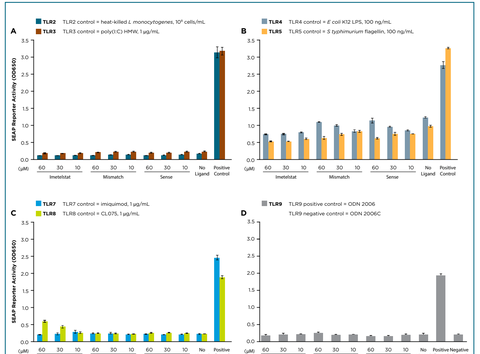

Figure 6. Imetelstat effects on TLR activity as measured by NFκB-inducible SEAP reporter assay

DISCUSSION

Proposed Mechanism of Action of Imetelstat in Patients With MPN

- Imetelstat affects normal megakaryopoiesis (**Figure 7**)[14] by delaying terminal maturation of megakaryocyte (MK) precursor cells and creating an accumulation of immature MK cells (**Figure 8**).[8]
  - Physiologic megakaryocytic differentiation requires upregulation of telomerase activity[14] and imetelstat inhibits telomerase activity.[8]
  - *Ex vivo* studies have provided evidence that the propensity for imetelstat to induce thrombocytopenia in patients with MPN results from imetelstat blocking the terminal maturation of normal MK precursors.[8]
  - Imetelstat inhibition of hTERT and telomerase activity is concurrent with effects on MK maturation.[8]
  - Reduction of the number mature MK cells with imetelstat treatment could then reduce production of platelets.
- Furthermore, by inhibiting telomerase activity, imetelstat treatment *in vitro* impairs MK polyploidization and morphologic maturation.[8]
- Imetelstat treatment *ex vivo* preferentially inhibits MK colony-forming units (CFU-MK) in samples from patients with MPN but not CFU-MK from healthy individuals (**Figure 9**).[8,15]
- Inhibition of telomerase and clonal proliferation of MK was also demonstrated in samples from patients with ET in the phase 2 study of imetelstat (**Figure 10** and **Table 1**).[1]

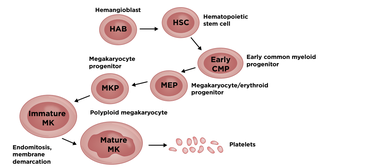

Figure 7. The megakaryocytopoietic developmental pathway[14]

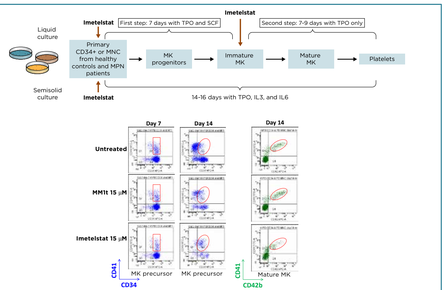

Figure 8. Imetelstat affects megakaryocytopoiesis *ex vivo*

A. Experimental design and method. Cells were suspended in QBSF-60 serum free-based MK differentiation medium (Quality Biological, Inc., Gaithersburg, MD) supplemented with 50 ng/mL thrombopoietin (TPO) and 50 ng/mL stem cell factor (SCF) for 7 days then allowed to mature for 7 additional days in fresh media supplemented with 50 ng/mL TPO only. **B.** Treatment with imetelstat during MK differentiation of CD34+ cells from healthy donors results in accumulation of immature $CD34^+/CD41^+$ MK and reduced number of mature $CD41^+/CD42^+$ MK, suggesting a delay in MK maturation. Flow cytometric analyses of Day 7 and Day 14 MK cultures generated in the absence (Untreated) and in the presence of the inactive (MM1) or active drug (imetelstat). MNC, mononuclear cells.

Figures included with permission.[8]

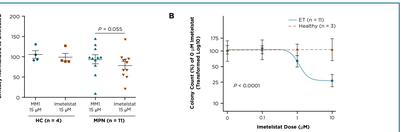

Figure 9. CFU-MK cells from patients with MPN are more sensitive to treatment with imetelstat than CFU-MK cells from healthy individuals

A. CFU-MK formation by peripheral blood mononuclear cells from healthy controls (HC, n = 4) and from patients with MPN (n = 11) grown in the presence of the inactive (MM1) or active drug (imetelstat).[8] **B.** Dose-response analysis in primary cells from patients with ET compared with healthy individuals.[15]

Figures included with permission.[8,15]

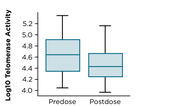

Figure 10. Telomerase is inhibited in patients with ET treated with imetelstat in a phase 2 trial[1]

Predose vs 24 hours postdose (7.5 mg/kg - 11.7 mg/kg) telomerase activity in 6 patients (3 with samples at 1 cycle and 3 with 2-3 cycles). Telomerase activity was reduced on average by 36% ($P < 0.001$ by pairwise permutation test).

Figure included with permission.[1]

Table 1. Imetelstat inhibits clonal cellular proliferation in patients with ET treated with imetelstat (7.5 mg/kg - 11.7 mg/kg) in a phase 2 trial, as measured by CFU-MK assay[1]

Patient number	Colonies at baseline (absolute number ± SEM)	Colonies at 1 month (absolute number ± SEM)
5	22.7 ± 0.7	1.7 ± 0.9
9	8.0 ± 1.6	0.3 ± 0.3
10	16.3 ± 0.7	8.1 ± 4.0
11	73.7 ± 7.0	6.0 ± 0.6
15	> 50	7.7 ± 1.9

SEM, standard error of the mean.

CONCLUSIONS

- Imetelstat, at a clinically relevant concentration range, had no stimulatory effect on the majority of tested TLRs.
  - The induction of TLR8 is not believed to be relevant because the induction was substantially lower than the positive control, and TLR8 has not been reported to be associated with thrombocytopenia.
- These results suggest that the thrombocytopenia observed in some patients treated with imetelstat is likely not driven via interactions with TLRs.
- These findings are supported by the structural differences between imetelstat and the minimal requirements to activate TLR9.
- It is instead hypothesized that the thrombocytopenia associated with imetelstat may result from on-target mechanisms. Other studies of imetelstat have demonstrated potential on-target mechanism for the observed thrombocytopenia.[1,8,15]
  - Telomerase inhibition in healthy megakaryopoiesis delays maturation of MK cells, thus reducing the number of MK available to produce platelets.
  - The inhibitory effects of imetelstat on CFU-MK are greater in samples from patients with ET compared with healthy individuals, suggesting a different mode of action of imetelstat in the regulation of telomerase in pathological versus normal cells.

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