In vitro and in vivo evidence of the steroid-sparing potential of afimetoran, an equipotent toll-like receptor 7/8 dual antagonist

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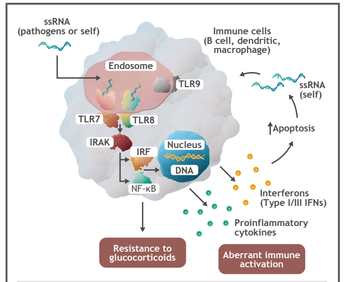
Introduction

- Systemic lupus erythematosus (SLE) is a highly heterogeneous chronic autoimmune disease, with glucocorticoid therapy as a standard of care
- Activation of toll-like receptors (TLRs) 7 and 8 plays a critical role in lupus disease biology, and their blockade leads to a significant reduction in lupus manifestations[1-3] (**Figure 1**)
  - TLR7/8 are important endosomal receptors involved in innate immunity, and their activation of nuclear factor kappa light-chain enhancer of activated B cells (NF-κB) contributes to steroid resistance[4]
- High-dose steroids are used frequently to control SLE disease activity. Long-term use can have serious side effects. Thus, a novel steroid-sparing therapeutic agent may markedly improve lupus management
- Previously we showed that afimetoran, an equipotent dual antagonist of TLR7/8 currently in clinical development for SLE treatment, demonstrates steroid-sparing effects with in vitro assays for apoptosis of human plasmacytoid dendritic cells (pDCs) and B cells, and in vivo in a New Zealand Black/White (NZB/W) mouse model of spontaneous lupus[4] (**Figure 2**)

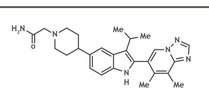
Figure 1. TLR7/8 in the pathophysiology of lupus



- TLR7/8 recognize single-stranded RNA (ssRNA) and initiate downstream signaling via the NF-κB and interferon (IFN) regulatory factor (IRF) pathways
- Lupus is associated with defects in clearance of apoptotic debris, which can activate TLR7/8
- Increased NF-κB signaling by TLR7/8 activation inhibits the steroid-induced apoptosis of immune cells, contributing to glucocorticoid resistance

IRAK, interleukin receptor-associated kinase.

Figure 2. Afimetoran is a highly selective and equipotent TLR7/8 dual antagonist



Afimetoran
(BMS-986256)

In vitro potency and selectivity assays		IC_{50} (nM)
HEK reporter	TLR7	0.65 ± 0.2
	TLR8	0.86 ± 0.5
	TLR9	6400 ± 2500
	TLR3/4	> 50,000
Human whole blood	TLR7 GDQ-induced IL-6	4.5 ± 0.4
	TLR8 TL8-506-induced IL-6	1.5 ± 0.1
Mouse whole blood[a]	TLR7 GDQ-induced IL-6	2.4 ± 1

[a]TLR8 is not functionally active in mice.
GDQ, TLR7-specific agonist (gardiquimod); HEK, human embryonic kidney; IL, interleukin; IC_{50}, half-maximal inhibitory concentration; TL8-506, TLR8-specific agonist.

Objectives

- This study provides additional insights into the cellular and molecular mechanisms underlying the steroid-sparing effects of afimetoran, both in vitro and in vivo
  - In vitro: assess IFN signature, chemokine/cytokine production, and resistance to prednisolone (PRED)-induced apoptosis in pDCs and B cells in whole blood (WB) samples from patients with SLE
  - In vivo: mouse models of SLE

Methods

In vitro

- WB samples or peripheral blood mononuclear cells (PBMCs) from patients with active SLE disease (SLE Disease Activity Index ≥ 6) were collected and treated with afimetoran (10 nM), PRED (1 ug/mL), or those in combination. Dimethyl sulfoxide (DMSO) served as a control. Four hours post-treatment, RNA was extracted from respective samples and subjected to RNA sequencing (RNAseq) analysis
- Following incubation, supernatants were analyzed for cytokines using the Luminex® platform and proportions of apoptotic B cells and pDCs were assessed using annexin V staining via flow cytometry

In vivo

- Afimetoran was evaluated in widely-accepted MRL/lpr, NZB/W-F1 and BXSB models of lupus nephritis (LN)
  - In all these models, mice were treated with vehicle or respective doses of afimetoran and PRED. Effect of treatment on kidney injury was assessed by measuring proteinuria
- Additionally, afimetoran alone and in combination with low-dose PRED was evaluated in BXSB mice, a model of spontaneous lupus and proliferative glomerulonephritis
  - Vehicle or selected doses of afimetoran and/or PRED (once daily) were tested
  - Kidney injury markers, serum titer of autoantibody such as anti-ribonucleoprotein (Ro) and plasma cytokine such as IL-12p40 were assessed in all treatment groups following 10 weeks of dosing
- In a separate experiment, to understand whether the effect achieved by combination of low-dose PRED and afimetoran is maintained by afimetoran alone, BXSB mice treated with the combination continued with afimetoran treatment alone for an additional 5 weeks
- For all the in vivo experiments, afimetoran was formulated in a vehicle of 10% ethanol, 45% polyethylene glycol 300, 5% PLURONIC® F68, and 40% 20 mM citrate buffer; PRED was formulated in a vehicle of 0.5% methyl cellulose, 0.2% Tween 80, and 99.4% water

Results

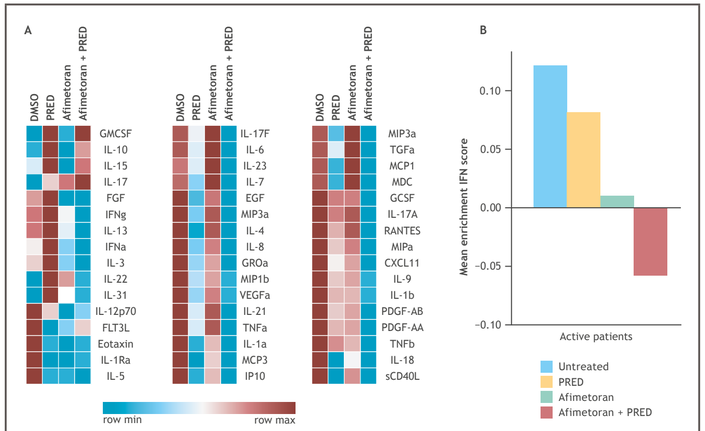
In vitro

- WB samples treated with afimetoran alone showed superior impact on multiple cytokines when compared with PRED. Combined treatment of afimetoran with PRED showed improved suppression of various cytokines when compared with PRED alone (**Figure 3**)
- A notable increase in PRED-induced apoptosis of pDCs and B cells was observed with afimetoran, compared with PRED alone or DMSO control (**Figure 4**)
  - TLR7 activation in patients with SLE generated resistance for PRED-induced apoptosis in B cells and pDCs
  - Upon blockade of TLR7 by afimetoran, PRED showed significant induction of apoptosis of B cells and pDCs

In vivo

- Afimetoran-treated mice showed significant suppression of kidney injury markers, plasma cytokines, IFN-secreting pDCs, and autoantibody titer compared with control mice ($P < 0.05$ to $P < 0.001$) (**Figures 5** and **6**)
  - Afimetoran combined with low-dose PRED showed higher suppression than either treatment alone (**Figure 6**)
- After the withdrawal of PRED, afimetoran alone maintained the benefits achieved by combination treatment in a mouse model of lupus (**Figure 7**)

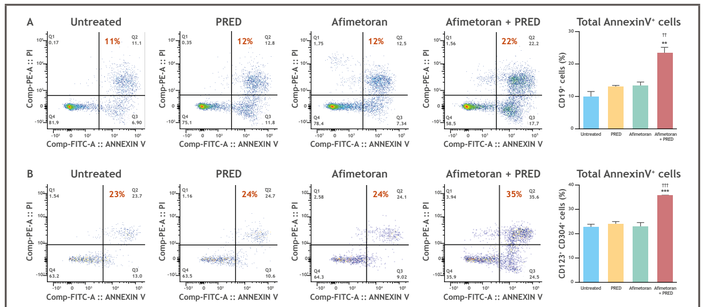
Figure 3. Afimetoran alone and in combination with PRED showed significant and greater impact than PRED alone on multiple chemokines and cytokines in samples from patients with SLE



- (A) WB of patients with SLE were treated with afimetoran, PRED, or afimetoran in combination with PRED. There was a > 50% reduction in multiple cytokines
- (B) Differentially expressed genes (DEGs) were identified via RNAseq analysis in WB samples from patients with SLE disease. Pathway enrichment analysis using DEGs revealed a superior impact of afimetoran compared with PRED on the IFN pathway enrichment score. Combination treatment showed further reduction in enrichment score of IFN pathway

Afimetoran tested at 10 nM (~2x IC_{50}) and PRED at 1 ug/mL. Follow-up testing with afimetoran at 3x IC_{50} is planned.
CXCL, chemokine (C-X-C motif) ligand; EGF, epidermal growth factor; FGF, fibroblast growth factor; FLT3L, FMS-related tyrosine kinase 3 ligand; GCSF, granulocyte colony-stimulating factor; GMCSF, granulocyte macrophage colony-stimulating factor; GRO, growth-related oncogene; IC_{50}, 90% of maximum inhibition; IP, interferon gamma-induced protein; MCP, monocyte chemoattractant protein; MDC, macrophage-derived chemokine; MIP, macrophage inflammatory protein; PDGF, platelet-derived growth factor; RANTES, regulated upon activation, normal T cell expressed and secreted; sCD40L, soluble cluster of differentiation 40 ligand; TGF, transforming growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

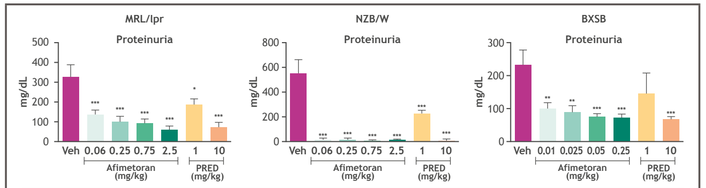
Figure 4. Afimetoran reversed resistance to steroid-induced apoptosis in (A) B cells and (B) pDCs of patients with SLE in vitro



- PBMCs from patients with SLE were treated in vitro with the indicated treatments. (A) B cells and (B) pDCs treated with PRED were not able to induce apoptosis of B cells and pDCs. In the presence of TLR7/8 blockade and through NF-κB inhibition by afimetoran, PRED significantly induced apoptosis of pDCs and B cells

**$P < 0.01$, ***$P < 0.001$ versus untreated; ††$P < 0.01$, †††$P < 0.001$ versus PRED alone.
One-way analysis of variance Dunnett's test. Percentages indicate the percentage of apoptosis.
PI, propidium iodide.

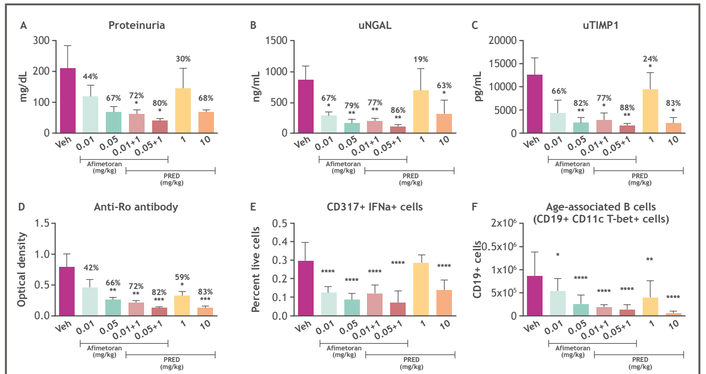
Figure 5. Afimetoran showed significant efficacy across three different mouse models of lupus



- Across all 3 mouse models, afimetoran showed a robust impact on kidney injury with significant reduction of proteinuria, a clinically relevant biomarker. Afimetoran showed similar efficacy to that of high-dose PRED across all 3 models

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus vehicle.
Veh, vehicle.

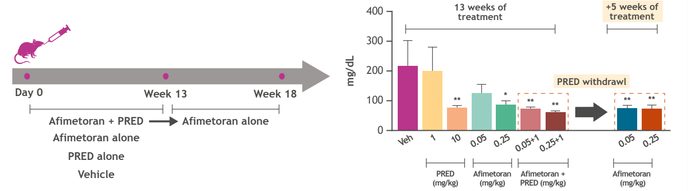
Figure 6. Afimetoran combined with PRED appeared to show improved anti-lupus/LN efficacy in BXSB mice



- Treatment of BXSB mice with afimetoran alone or in combination with PRED showed robust efficacy with significant suppression of (A) proteinuria, (B) uNGAL, and/or (C) uTIMP1 compared with vehicle or high-dose PRED alone. Similar trends were observed in (D) anti-Ro antibodies, (E) proportion of pDCs secreting IFNα, and (F) age-associated B cells (ABCs)

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ vs vehicle.
uNGAL, urinary neutrophil gelatinase-associated lipocalin; uTIMP1, urinary tissue inhibitor of metalloproteinase 1.

Figure 7. Afimetoran demonstrated the potential to replace PRED in a BXSB mouse model of lupus



- Continuation of afimetoran treatment alone for an additional 5 weeks, after withdrawal of PRED from combination treatment for 13 weeks, was able to maintain a similar level of robust efficacy comparable to high-dose PRED

*$P < 0.05$, **$P < 0.01$.

Conclusions

- Afimetoran, a highly selective TLR7/8 dual antagonist, showed robust effect in cells from patients with SLE and strong steroid-sparing potential in mice
  - In the in vitro setting:
    - Afimetoran reversed resistance to steroid-induced apoptosis in B cells and pDCs from patients with SLE
    - Afimetoran alone showed significant and greater impact than PRED alone on multiple chemokines and cytokines in WB samples from patients with SLE. Combination treatment further improved this effect
    - Transcriptomic analysis revealed a far superior impact of afimetoran than PRED on the IFN pathway signature in WB samples from patients with SLE. Combination treatment displayed an even stronger impact than afimetoran or PRED monotherapies
- In vivo, afimetoran is robustly effective against lupus and LN across 3 mouse models of lupus (MRL/lpr, NZB/W, BXSB)
  - In the BXSB model of lupus:
    - Afimetoran combined with PRED appeared to show improved efficacy against lupus/LN compared with either treatment
    - After withdrawal of PRED, afimetoran alone maintained efficacy similar to combination treatment
    - These results support the potential of afimetoran to replace steroids for the treatment of lupus and LN

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Disclosures

SD, PC, BK, KC, AD, QZ, and FB are employees of and hold stock or stock options in Bristol Myers Squibb. SSu, AR, SP, NSB, VP, SSe, MS, and AA are employees of Biocon Bristol Myers Squibb Research Center.