Liver and Splenic Clearance of Platelets Following Treatment of Cynomolgus Monkeys with ISIS 104838 is Accompanied by Innate Immune Activation

POSTER # P565

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CONFLICT OF INTEREST STATEMENT
All of the authors listed are full time employees of the affiliations as indicated

ABSTRACT (modified)

ISIS 104838 is representative of a subset of 2'-*O*-methoxyethyl (2'-MOE) modified ASOs that cause moderate dose-dependent decreases in platelet counts (PLT) in nonhuman primates (NHPs). Bone marrow toxicity, splenomegaly, direct ASO-mediated complement activation, and thrombosis have been excluded as mechanisms contributing to this PLT decrease. In a recent investigative study, five NHPs were treated with 60 mg/kg/wk ISIS 104838 by subcutaneous administration for 2 weeks followed by a maintenance dose of 30 mg/kg/wk to produce moderate PLT reductions. Before treatment and after 8 weeks of treatment, NHPs were injected with ^{111}Indium-oxine labeled PLTs in order to evaluate PLT distribution/clearance. Additionally, serum total immunoglobulins (Ig), cytokines, chemokines, inflammatory mediators (e.g. von Willebrand Factor (vWF)), growth factors (e.g., thrombopoietin (TPO)), and anti-PLT or anti-platelet factor 4 (anti-PF4) antibodies were also measured. Animals had moderate reductions (~50%) in PLT levels by Day 30 compared to their pre-dose values, ranging from 396 x10^3 to 551 x10^3 cells/µL. A mild to moderate increase (25–45% of total cumulative counts) in PLT sequestration in liver and spleen was evident for animals with moderate PLT reductions. Concomitant increases in total serum IgM (2- to 4.5-fold increase) relative to baseline values, was maintained with continued dosing. This was associated with increases in anti-PF4 IgM and anti-PLT IgM antibodies (~2 to 5-fold increase compared to baseline) and was seen in all but one animal, which had a high baseline level of anti-PF4 IgM. This effect was consistent across cynos and followed a mirror image of PLT reduction. There was no increase in HIT-causing anti-PF4 IgG antibodies. Amongst the many cytokines, chemokines and inflammatory mediators evaluated, only MCP-1 showed an increase which occurred in all cynos, and is a consistent feature of innate immune cell activation by non-CpG MOE ASOs (Younis, Vickers et al. 2006). Although more investigation is needed, these results suggest that increases in anti-PLT IgM and/or anti-PF4 IgM, secondary to immune cell activation, could lead to an increased rate of PLT sequestration in NHPs.

BACKGROUND

- A limited subset of 2'-MOE sequences caused consistent decline in PLT count in nonhuman primates (NHPs)
- Moderate decreases in PLT count observed with approximately 30% of ASO sequences is a dose-dependent and reproducible 30-50% decline from baseline
- Not considered adverse for the animals because the PLT counts do not generally decrease below 75 x10^3 to 100 x10^3 /µL, with group mean values near 150 x10^3/µL
- Translatable to humans; prototypical ASO that manifests this phenotype is ISIS 104838
- Prior investigations have excluded bone marrow toxicity, splenomegaly, direct ASO-mediated complement activation, megakaryocyte homeostasis, thrombopoeitin deficiency, and thrombosis among other mechanisms
- The exact mechanism of moderate PLT declines is currently unknown, but the recent study described here seem to point towards an effect of the ASO on immune cells resulting in enhanced clearance of PLTs, sans PLT activation

Various End Points for Mechanistic Evaluation

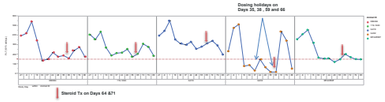
	-21	-9	1	4	7	10	14	16	17	21	24	28	30	31	35	37	38	44	45	48	52	55[*]	59	62	66	72	73	79	80	86
PLT Sequestration		X																				X								
Hematology	X	X	X						X				X		X		X		X		X		X		X		X		X	
Clinical Chemistry	X																													X
Microparticle	X							X					X		X		X		X		X						X			X
ADAMTS13	X							X					X		X						X						X			X
vWF	X												X								X						X			X
Glycocalicin	X							X					X		X						X						X			X
IgG/IgM	X												X								X						X			X
Anti-PLT / PF4	X												X								X						X			X
Cytokine	X												X								X						X			X
TPO	X												X								X						X			X
Phagocytosis	X												X								X						X			X
PBMC																								X						X

* Gray highlight indicates dose days
# Collection prior to steroid treatment

- End points discussed in this poster:
  - Clinical Pathology
  - PLT sequestration with ^{111}In oxine-labeled autologous PLT (BioLaurus)
  - PF4 and PLT antibodies (BCW)
  - PLT-derived microparticles
  - Cytokines/chemokines/endothelial factors

FINDINGS

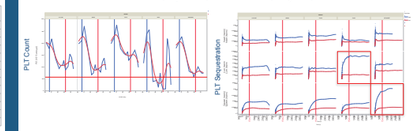
Consistent, Reproducible PLT Decrease in Female Cynomolgus Monkeys Given ISIS 104838



- 30 mg/kg on Days 1, 3, and 7 followed by twice weekly SC dosing
- Day 16 - Animal G432 - severe thrombocytopenia, with increased Mean Platelet Volume, following 5 doses - 78 x10^3/µL
  - PLT recovery after dosing holidays (148 x10^3/µL on Day 44)
  - Drop on re-challenge (16 x10^3/µL) on Day 55
- Day 30 - 4/5 animals had a reduction in PLT count ~ 150 x10^3/µL
- Day 45 - dose reduction (from 60 to 30 mg/kg/wk) for all animals to maintain reduced PLT count
- Days 64 and 71 - Steroid treatment (Depo-Medrol, 80 mg/1st week, 40 mg/2nd week).
  - Only Animal G432 responded to steroid treatment, with PLT counts rebounding to prestudy baseline values but was only able to maintain the level for less than two weeks
  - All other 4 monkeys had minimal to no response to steroid treatment

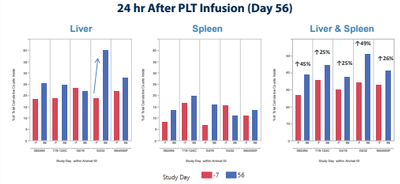
^{111}In-PLT Labeling to Evaluate Platelet Sequestration in Spleen, Liver & Heart in Cynomolgus Monkeys On Days -9 and 55

Dynamic Imaging Shows ASO Treatment Exacerbates Consistent and Rapid PLT Distribution to Liver, Spleen, and Heart (Day 55)

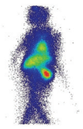


- All animals exhibit about 50% PLT decrease by D55 except G432 which experienced a severe reduction
- PLT (imaging – lower graph) was normalized to Dose(Cpm)/Body Weight for each time point and animal
- Most PLT sequestration occurs very rapidly and within 10-15 minutes PLT levels in each organ has reached steady state
- All animals exhibited a higher level of organ sequestration on D55 compared to Day -8
  - Increased PLT sequestration in the liver for G432 correlated with overall decrease in PLT count
  - PLT sequestration prior to ASO dosing is comparableacross animals

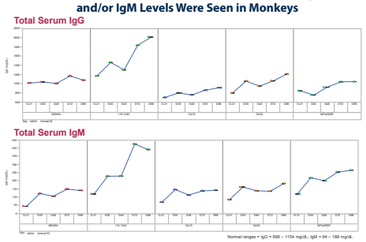
Static Imaging Shows an Increase in Cumulative ^{111}In-PLT (Count or Gamma Photon) in Liver and Spleen Was Evident in All Animals 24 hr After PLT Infusion (Day 56)



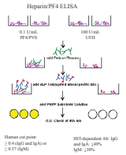
- There were mild to moderated increases (25–45%) in PLT sequestration from baseline in liver and spleen combined on Day 56 for animals with moderate PLT reduction
- A 49% increase in combined PLT sequestration (driven by increased liver sequestration) was also seen in Animal G432 with severe thrombocytopenia
  - Cumulative ^{111}In-PLT counts for liver, spleen and heart were collected over approximately 1000 image seconds, except for G432 on Day 56 which were collected over 2208 seconds due to low PLT counts
  - Data is presented as % of total cumulative counts inside the body



Minimal to Moderate Increases in Total Serum IgG and/or IgM Levels Were Seen in Monkeys

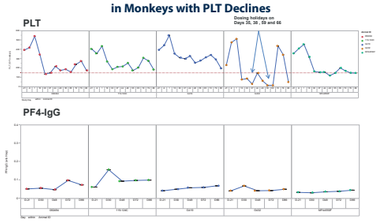


Detection of Anti-PF4/Heparin Antibodies in Serum By ELISA

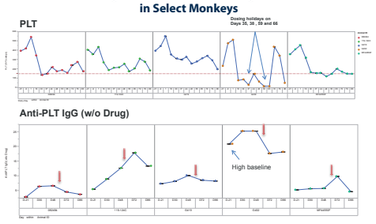


- Monkey serum was incubated in plates pre-coated with complexes of PF4-PVS with and without the presence of 100 U/mL heparin
  - Polyvinylsulfonate (PVS) is a polyanionic compound which is used as a substitute for unfractionated heparin
- Alkaline-phosphatase labeled anti-human IgG or IgM was used as a secondary antibody
- O.D. values greater than the human cut point that inhibited by at least 49% for IgG or 30% for IgM in the presence of heparin (100 U/mL) are considered positive for this assay
  - Human cut points are not used for evaluation of the monkey sample results

No Increase In Anti-PF4-IgG Antibody Was Observed in Monkeys with PLT Declines

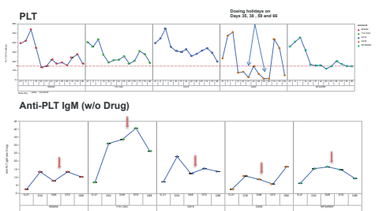


Increased Drug-independent Anti-PLT IgG was Observed in Select Monkeys



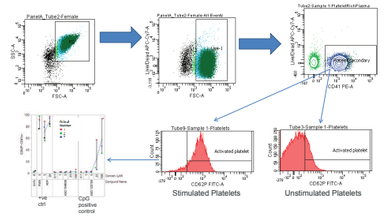
Increased Anti-PF4-IgM Levels From Baseline Were Seen in Select Monkeys With Moderate PLT Reduction

Increased Drug-independent Anti-PLT IgM was Observed In Select Monkeys with Moderate PLT Reduction



ASSESMENT OF MONKEY PLATELET ACTIVATION *IN VITRO* AND *IN VIVO*

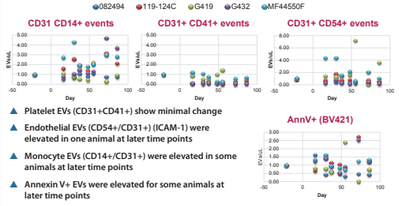
ISIS 104838 Did Not Activate Monkey Platelets *In Vitro*



Detection of Extracellular Vesicles (EVs) in Monkey Plasma: *In Vivo* Assessment of Platelet EVs as Markers of Platelet Activation

- Samples were diluted and stained (in duplicate) with vesicle staining solution and a fluorescent antibody cocktail consisting of
  - CD54-DyLight488 (endothelial cells)
  - CD61-PE and CD41-APC (platelets)
  - CD31-BV421 (PECAM or P-Selectin)
  - CD14-BV605 (monocytes)
  - Annexin V-BV421 (apoptosis)
- PECAM-1 is found on the surface of platelets, monocytes, neutrophils, and some types of T-cells, and was used in conjunction with CD14, CD54, and CD41 to separate out PLT, monocytes and endothelial cells
- Controls included Buffer only, a Reference Vesicle containing phosphatidylserine and no antigen (which serves as a positive control for Annexin V binding and a negative control for antibody binding)
- Calibrated capture beads (which serve as a positive control for antibody binding), a normal human platelet-poor plasma (PPP) preparation and a normal human platelet-rich plasma (PRP)-derived preparation

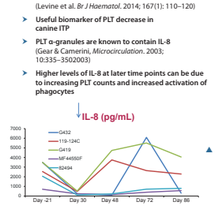
An Increase in Endothelial-derived, Monocyte-derived (CD14), and Apoptotic Microparticles (Annexin V) was Observed in Select Animals



- Platelet EVs (CD31+CD41+) show minimal change
- Endothelial EVs (CD54+/CD31+) (ICAM-1) were elevated in one animal at later time points
- Monocyte EVs (CD14+/CD31+) were elevated in some animals at later time points
- Annexin V+ EVs were elevated for some animals at later time points

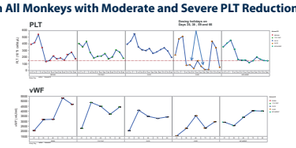
ISIS 104838 Induces Variable Response in Chemokines Using a Mulitplex Assay

- IL-8 levels decrease as PLT count decreases
  - PLTs have been previously described as a significant source of IL-8 in canine serum (Levine et al. *Br J Haematol*. 2014; 167(1): 110–120)
  - Useful biomarker of PLT decrease in canine ITP
  - PLT α-granules are known to contain IL-8 (Gear & Camerini, *Microcirculation*. 2003; 10:335–3502003)
  - Higher levels of IL-8 at later time points can be due to increasing PLT counts and increased activation of phagocytes



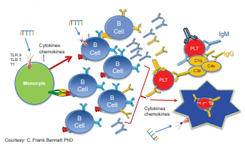
- Increased plasma concentration of inflammatory chemokines, such as MCP-1, has been observed after 2'-MOE ASO administration and is likely contribute to downstream secondary inflammatory responses seen with this class of ASOs

Increase in von Willebrand Factor (vWF) Levels were Observed in All Monkeys with Moderate and Severe PLT Reduction



- vWF is a large multimeric glycoprotein produced in endothelium (in the Weibel-Palade bodies), PLT α-granules, and subendothelial connective tissue
- vWF plays an essential role in supporting PLT adhesion and activation
- Activity of ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13), which regulates the multimeric size of VWF, was not inhibited in these animals, (data not shown)

Schematic Representation of Direct B-Cell and Monocyte Activation by ISIS 104838 Leading to Polyclonal Memory B cell, anti-PLT IgM, anti-PLT IgG Responses



Pre-existing anti-PLT Auto IgG, complement activation, and enhanced innate immune response leads to immune complex formation on the platelet surface leading to macrophage clearance in spleen and liver

SUMMARY

- Consistent, reproducible PLT decrease in all monkeys with no increase in MPV
  - 1 out of 4 animals had severe thrombocytopenia
- No direct PLT activation *in vitro* or *in vivo*
  - Lack of CD62P upregulation and circulating PLT EVs
- Increased (25–49%) PLT sequestration in liver and spleen
  - Consistent PLT decreases in 4/5 monkeys were insensitive to steroid treatment
  - Severe thrombocytopenia in one monkey (G432) was responsive to steroid treatment
  - No indication of desialylation (data not shown)
  - Sequestration could be driven by PLT bound IgM/complement or IgG
- Increased neutrophil and monocyte activity leading to increased chemokines
- Increase in vWF observed in all monkeys, without inhibition of ADAMTS13 activity
  - Source of vWF not fully ascertained
  - Increased vWF could be either a cause of PLT reduction, consequence of PLT activation and/or endothelial activation
- Evidence of immune-mediated mechanism (anti-PF4-IgM and/ anti-PLT IgG/M)
  - General increase in total plasma IgG/IgM and MCP-1
  - No apparent anti-PF4 IgG antibodies
  - Tendency for anti-PF4 IgM and anti-PLT IgM/IgG to be elevated from baseline
    - Most pronounced in 1 out of 5 monkeys with moderate PLT reduction
  - The high baseline drug-independent anti-PLT IgG in monkey G432 likely predisposed this monkey to the rapid severe reduction with ASO treatment

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