No mutations detected with PMO*plus* antisense oligomers that protect nonhuman primates against marburgvirus

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ABSTRACT

Objectives: *Marburgvirus* (MARV) is highly virulent RNA virus of the family *Filoviridae* and a causative agent of viral hemorrhagic fever (VHF). The postexposure therapeutic efficacy of AVI-6003 [a PMO*plus* combination targeting MARV nucleoprotein (NP, AVI-7288) and VP24 (AVI-7287)] has reproducibly provided for 100 percent survival in nonhuman primate (NHP) MARV lethal challenge infections. The objective was to evaluate the viral sequence fidelity in viral genome regions targeted by AVI-6003 in nonhuman primates infected with MARV Musoke in treated versus untreated groups.
Methods: Three independent studies were conducted in which cynomolgus macaques were infected with 1000 pfu of MARV Musoke. Five groups of infected NHPs included daily doses of AVI-6003, AVI-7287 alone, AVI-7288 alone, a negative-control PMO*plus* agent, and saline. Viral genomic RNA was obtained from infected animals immediately upon sample collection by mixing one volume of serum with three volumes of Trizol LS. Samples with > 300 pfu/mL were amplified using random hexamer DNA primers and reverse transcriptase PCR amplification. Viral genome sequence was determined using dye-terminator (studies 1 and 2) and pyrosequencing (study 3) methods.
Results: The amplicon DNA sequence was determined to an average depth of up to 3300 for the NP site and 6700 for the VP24 site including an approximate 200 base flanking region. Determination of the entire viral genome sequence is currently in progress. The sequence observations encompass 3 independent studies, evaluating blood or serum samples from 35 different NHPs (29 treated at doses from 7.5 to 40 mg/kg/day and 6 saline controls), and samples were evaluated from days 8 to 30 post infection. The PMO targeted viral genome sites were found to demonstrate high fidelity and show no sequence changes indicating no development of resistanceto AVI-6003.
Conclusions: These results indicate viral resistance to AVI-6003 and its components is unlikely in the genome of this single-stranded RNA human pathogen and support the further development of PMO*plus* therapies for use in humans.

INTRODUCTION

Lake Victoria Marburgvirus (MARV) is a filamentous, single-stranded, negative-sense RNA virus of the family *Filoviridae* that can cause a rare human hemorrhagic fever (MHF). No established effective therapy is available to treat or prevent any of the filovirus infections. Treatment is supportive. Various experimental interventions have been preliminarily evaluated, including fusion inhibitors, transcription/replication inhibitors, maturation inhibitors, small interfering RNA, antibody therapy, inflammatory modifiers and coagulation modulators, but, in general, *in vivo* benefits have not been documented for these experimental agents.

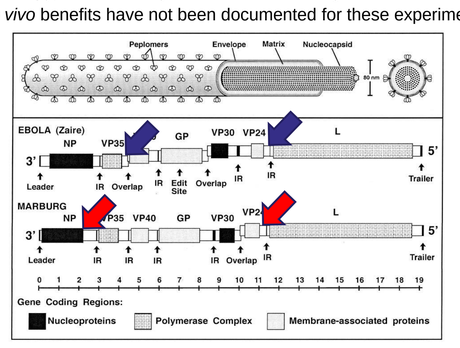
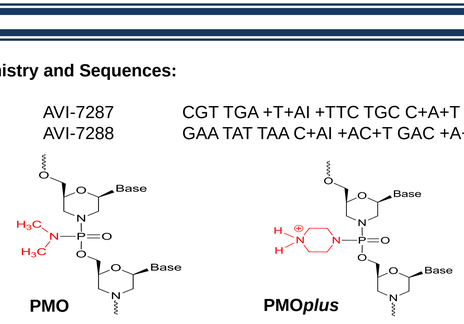


Figure 1. Oligomer target sites (blue for AVI-6002 and red for AVI-6003). Two sites were selected for each virus to reduce the probability of viral mutation resulting in resistance.

AVI BioPharma has developed a proprietary adaptable platform chemistry, phosphorodiamidate morpholino oligomers (PMO), that have significantly improved the stability, efficacy, specificity, delivery, and safety of antisense complexes. AVI-7288 is a PMO*plus* oligomer that binds directly to viral RNA of the nucleoprotein (NP) transcript with a binding equilibrium constant of between 6.5 10^{-12}M. The MARV particle is composed of 7 structural proteins. Four of them, NP, VP35, VP30 and L, form the nucleocapsid complex that surrounds the viral genome. The nucleocapsid protein, NP, is detected in 2 forms in infected cells (92 and 94 kDa). The 94 kDa form, a phosphorylated protein, appears to interact with VP35, which binds to the RNA-dependent RNA polymerase L and is essential for transcription and replication of the viral RNA genome. The non-phosphorylated, 92 kDa, form of NP is thought to bind strongly to viral RNA leading to encapsidation of viral RNA. Phosphorylation of NP occurs in region II, amino acids 439 – 475, of the C-terminal portion of NP occurs in an overlapping region with the RNA binding site of the protein, amino acids 289 – 352. Phosphorylation of NP leads to weaker binding of NP to RNA, due to repulsion of negatively charged RNA to the negative phosphate groups, resulting in limited access of the polymerase complex to the RNA template. These data suggest NP is a molecular switch which in the phosphorylated form facilitates transcription and replication necessary for the early portion of the viral life-cycle and a non-phosphorylated form that serves to encapsidate the viral RNA genome in preparation for viral egress from an infected cell. AVI-7288 is designed to inhibit NP synthesis resulting in catastrophic effects on production of viral mRNA, viral genome replication, and viral assembly.

AVI Chemistry and Sequences:

AVI-7287 CGT TGA +T+AI +TTC TGC C+A+T IC+T
AVI-7288 GAA TAT TAA C+AI +AC+T GAC +A+AG TC



METHODS

Studies were designed to evaluate the viral sequence fidelity in viral genome regions targeted by AVI-7288 in nonhuman primates infected with MARV Musoke in treated versus untreated groups. Three independent studies were conducted in which cynomolgus macaques were infected with 1000 pfu of MARV Musoke. Five groups of infected NHPs included daily doses of AVI-6003, AVI-7287 alone, AVI-7288 alone, a negative-control PMO*plus* agent, and saline. Viral genomic RNA was obtained from infected animals immediately upon sample collection by mixing one volume of serum with three volumes of Trizol LS. Samples with > 300 pfu/mL were amplified using random hexamer DNA primers and reverse transcriptase PCR amplification. Viral genome sequence was determined using dye-terminator (studies 1 and 2) and pyrosequencing ("deep sequencing," study 3) methods. The amplicon DNA sequence was determined to an average depth of up to 116,000 for the NP site and the flanking region. The sequence observations encompass 3 independent studies, evaluating blood or serum samples from 35 different NHPs (29 treated at doses from 7.5 to 40 mg/kg/day and 6 saline controls), and samples were evaluated from days 8 to 30 post infection. The PMO targeted viral genome sites were found to demonstrate high fidelity and show no sequence changes indicating no development of resistance to AVI-7288.

This work was conducted under contract with the Department of Defense Joint Project Manager Transformational Medical Technologies.

Conclusions:

- The Marburg Musoke viral genome sequence does not accumulate mutations in AVI-7287 or AVI-7288 drug binding region.
- A PMO*plus* oligomer targeting NP (AVI-7288) is effective against Marburg Musoke lethal challenge in nonhuman primates.
  - Observe 100 percent survival (10/10 in PMO 14) in AVI-7288 treatment group compared to zero survival (0/5) in the untreated group ($p < 0.05$).
  - AVI-7288 also effectively reduces viremia.
- Viral resistance to AVI-7288 is unlikely in the genome of this single-stranded RNA human pathogen and support the further development of PMO*plus* therapies for use in humans.
- AVI-7288 represents the optimal candidate for the prophylaxis and treatment of Lake Victoria Marburgvirus infections.

Study PMO 9: AVI-6003 in Marburgvirus

Dose	Number	Route of Administration	Day of Death	Survivors at 28 Days
PBS Control	1	s.c. + i.p.	D8	0/1
AVI-6003* @ 40 mg/kg	3	s.c.	-	3/3
AVI-6003 @ 30 mg/kg	3	s.c. + i.p.	-	3/3
AVI-6003 @ 40 mg/kg	3	i.v.	-	3/3

*AVI-6003 is a combination of AVI-7287 (NP) and AVI-7288 (VP24)

NHP Study #, treatment	Animal ID	Day Post challenge	Animal Fate	Direct Analysis		Plaque Analysis[1]	
AVI-6003: MARV in Cynomolgus macaques				NP	VP24	NP	VP24
9, AVI-6003	C0204013	8	Survived	No Mut.	No Mut.	No Mut. (10)	No Mut. (10)
9, AVI-6003	A15652	8	Survived	ND	ND	ND	ND

[1]Number in parenthesis indicates number of plaques tested.

Study PMO11: AVI-6003 in Marburgvirus

Dose (intravenous)	Number	Gender	Day of Death	Survivors at 28 Days
PBS Control	1	M	D11	0/1
Scr AVI-6002	4	M	1-D9, 2-D10, 1-D12	0/4
7.5 mg/kg	5	M	1-D14, 1-D16	3/5
15 mg/kg	5	M	1-D14, 1-D16	3/5
30 mg/kg	5	M	1-D12*	4/5*

*One animal died from a reaction to anesthesia.

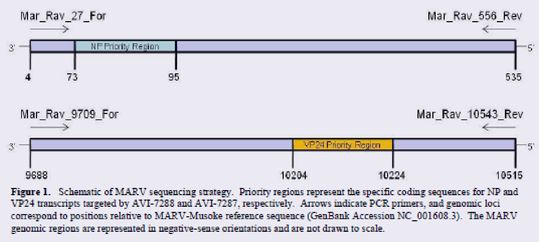


Figure 1. Schematic of MARV sequencing strategy. Priority regions represent the specific coding sequences for NP and VP24 transcripts targeted by AVI-7288 and AVI-7287, respectively. Arrows indicate PCR primers, and genomic loci correspond to positions relative to MARV-Musoke reference sequence (GenBank Accession NC_001608.3). The MARV genomic regions are represented in negative-sense orientations and are not drawn to scale.

Sequence aligning with at least a portion of MARV-Musoke reference sequence was obtained for all samples:
1. NP priority region was determined at a depth of 2-6X for 97% of samples (37 of 38)
2. VP24 priority region was determined at a depth of 1-6X for 79% of samples (30 of 38)

Conclude: **No mutations detected in binding region**

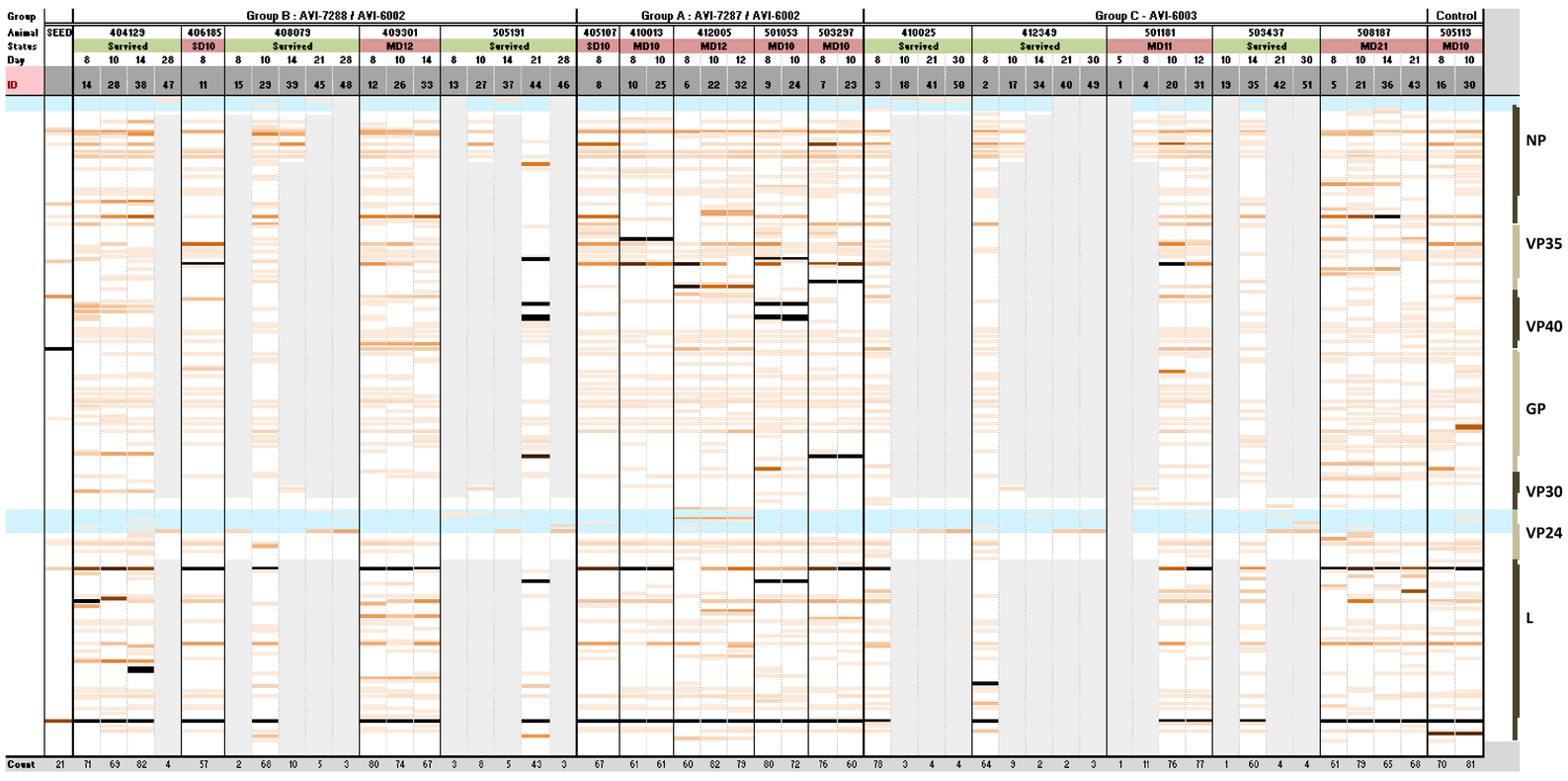
Study PMO12: AVI-6003 in Marburgvirus

Dose (intravenous)	Number	Day of Death	Survivors at 28 Days
PBS Control	1	1-D12	0/1
AVI-7287 @ 7.5mg/kg AVI-6002 @ 7.5mg/kg	5	1-D10, 1-D11	1/5
AVI-7288 @ 7.5mg/kg AVI-6002 @ 7.5mg/kg	5	4-D10, 1-D10	3/5
AVI-6003 @ 15mg/kg	5	1-D10, 1-D21	3/5

Sequencing Report	
Total Samples	51
Full Genome	30 samples + 1 seed stock
PMO Sites 1 and 2	12 samples
PMO Site 2 only	8 samples
No sequence	1 sample (Day 5, 501181)
Avg Depth	116,761

Sequence strategy:
1. Reverse transcription using random hexamers and UGC-24, which anneals to the 3' end of the MARV genome
2. First strand cDNA amplified with 12 primer pairs.
3. SISPA (sequence independent single primer amplification) amplification using random hexamers to produce sequence libraries. Methods were compared.
4. Sequence platform is Illumina GAIIx. Select 76 bp reads for optimal length/fidelity

Viral Genome Sequence Heat Map



Mutations
Total: **7**
Synonymous: **5**
Non-synonymous: **2 arrows**

Mutation frequency correlated with historical passage drift in other Filovirus seeds.
Low genetic diversity at point of interaction between therapeutic and viral genome.

SNP detection 1% = white
SNP detection >5% = orange
SNP occurring >50% = dark orange

Study PMO 14: AVI-7288 in Marburgvirus

Group	Treatment Route	Test/ Control	Regimen	Total Daily Dose	Survival
1	IV	15 mg/kg AVI-7287 15 mg/kg AVI-7288	SID: D0-D13	30	9/10
2	IV	15 mg/kg AVI-7288	SID: D0-D13	15	10/10
3	IV	0.9% Saline	SID: D0-D13	NA	0/5