

An Emerging Therapeutic for the Treatment of Dengue Viral Infections

Patrick L. Iversen, Ph.D.¹, Stacey Crumley¹, Dan V. Mourich, Ph.D. ¹ and Tom Voss²

¹AVI BioPharma, Bothell, WA, and Corvallis, OR, USA , ²Tulane School of Medicine, New Orleans, LA



Background: Dengue virus infections result in a spectrum of disease, ranging from Dengue fever (DF), to Dengue hemorrhagic fever (DHF) and Dengue shock syndrome (DSS). Earlier studies utilized a phosphorodiamidate morpholino oligomer (PMO) or peptide conjugated PMO to identify the 5'-stem loop (5'-SL) and the 3'-cyclization sequence (3'-CS) as highly conserved viral targets.

Methods: In vivo efficacy studies were conducted with AVI-6006, a combination of the 5'-SL and 3'-CS oligomers, in AG129 mice infected intravenously with the DEN2 S221 viral strain which causes early lethal disease in mice, including increased vascular leakage and cytokine storm. In addition, efficacy studies were conducted in ferrets. The endpoints of the studies included median survival time (MST), long term survival, body weight changes and blood and tissue virus load. The studies evaluated PMO, peptide conjugated PMO and PMOplus chemistry for the 5'-SL and 3'-CS sequences.

Results: AVI-6006 provided greater potency relative to the PMO and less toxicity than the PPMO (peptide conjugated PMO) oligomers composed of the same 5'-SL and 3'-CS target sequences. In the mouse a 3.0 mg dose of PMOplus oligomers administered by the intraperitoneal route on days 0, 1, 2, 4, 6 and 8 post infection provided for MST of 14 days and 20 percent long term survival versus MST of 4 days and no long term survival in the saline control and the scramble PMOplus control sequence (p<0.05). In the ferret, a dose of 150mg/kg (75mg/kg each of 5'-SL and 3'-CS) was well tolerated and reduced body weight loss from infection.

Conclusions: AVI-6006 oligomers targeting the 5'-SL and the 3'-CS are effective against Dengue 2 in both the mouse lethal challenge and ferret challenge models providing survival benefit, reduction in viral titer and prevention of body weight loss. The 5'-SL and 3'-CS combination provides a broad separation in the effective dose from the adverse event dose.

INTRODUCTION:

The *Flaviviridae* family is defined by a single-stranded RNA genome with positive sense polarity. There are over 70 different viruses in the family which gets its name from the Latin, *flavis* or yellow, which comes from the Yellow Fever Virus discovered by Walter Reed. Dengue is a member of the *Flaviviridae* family and contains four distinct serotypes. Dengue virus (DENV), transmitted by mosquito bites, results in a spectrum of disease from no symptoms to Dengue Fever (DF) and Dengue Hemorrhagic Fever (DHF) which can be fatal. Severe and fatal disease is more associated with DENV-2 and DENV-3 serotypes. The CDC estimates 50 to 100 million people are infected with dengue virus each year with slightly less than 1 million cases of DF/DHF. The WHO estimates that DF and DHF together result in 500,000 hospitalizations and 15,000 deaths per year. Therefore, dengue virus is an important pathogen to the nearly 2 billion people that live in endemic infection areas of the world and those that travel to those endemic regions.

RNA therapeutic approaches to DENV therapeutics have been reported in the peer reviewed literature. Antisense phosphorothioate oligonucleotides containing C-5 propyne bases were reported effective in tissue culture and RNA interference (RNAi) delivered by adeno-associated virus was also effective in cell culture. No subsequent reports following these leads appear in the literature. We investigated five different regions of DENV using phosphorodiamidate morpholino oligomers (PMO) designed to bind to the 5'-stem loop (5'-SL), the single AUG translation initiation start site, the 5'-cyclization site (5'-CS), the 3'-cyclization site (3'-CS), and the 3'-stem loop (3'-SL). These studies identified two highly conserved regions, the 5'-SL and the 3'-CS, which could reduce viral titer in cell culture by more than 5 logs and were effective against all four DENV serotypes (Kinney et al., 2005). Detailed investigation into the role of the 3'UTR in DENV replication involved five highly conserved regions in the 3'UTR as well as the 5'-SL and the AUG translation initiation site. These studies revealed the 3'-CS PMO inhibits viral replication while the 5'-SL inhibits both viral replication and translation (Holden et al., 2006). These studies led to the current studies which involve in vivo efficacy studies with the 5'-SL and 3'-CS sequences.

Studies to refine the optimal length of the peptide conjugated PMO (PPMO) for both 5'-SL and 3'-CS were conducted in cell culture (Stein et al., 2008). A series of PPMO with length 14 to 30 were evaluated at a constant 1 uM concentration in Vero cells infected with DEN-2 at MOI of 1.0 pfu/cell. The 22-mer lengths were consistently highly effective for both the 5'-SL and the 3'-CS. This observation is interesting in that binding intensity for longer molecules is greater and does not show direct correlation with antiviral activity in culture.

References:

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METHODS

Animals. The laboratory of S. Shrestha at the La Jolla Institute for Allergy and Immunology (LIA) identified a viral strain, D2510 that causes disease in mice which includes increased vascular permeability, paralysis and death (Shrestha et al., (2006). The first experiment involved 10 groups of 5 mice in each group. The S221 viral isolate of the D2510 strain was administered to mice (1 x 10⁶ pfu) by intravenous tail vein injection. The PMO and PMO compounds were administered at 4hrs, +20hrs, 2, 4, 6 and 8 days after infection by the intraperitoneal route.

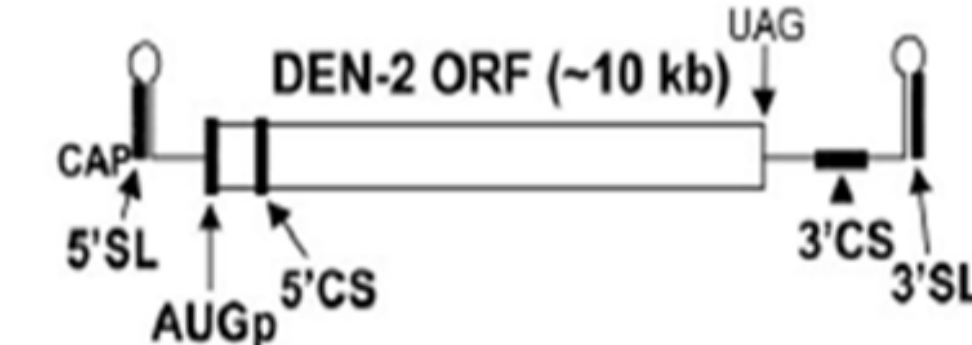
The second experiment involved 12 groups of 5 mice in each group. The S221 viral isolate of the D2510 strain was administered to mice (2 x 10⁶ pfu) by intravenous tail vein injection. The viral burden was twice that in experiment 1 which was selected to ensure a reliable and aggressive infection. The PMO and PPMO compounds were administered at 4hrs, +20hrs, 2, 4, 6 and 8 days after infection by the intraperitoneal route. The infection inoculums were 2 x 10⁶ pfu/mouse administered at time 0 which is twice the pfu in study 1.

The third experiment involved 9 groups of 5 mice in each group. The S221 viral isolate of the D2510 strain was administered to mice (2 x 10⁶ pfu) by intravenous tail vein injection. PPMO compounds were administered at -4hrs, +20hrs, 2, 4, 6 and 8 days after infection by the intraperitoneal route. The purpose of the study was to compare increasing doses of both single 5'-SL PPMO with the combination of 5'-SL and 3'-CS PPMOs. Based on the observations in experiment 2, the "B" peptide and the 5'-SL-22 mer were selected for this dose-response study.

The fourth experiment involved seven groups of 8 mice per group (56 mice total) with 5 mice from each group for the survival endpoint and 3 mice from each group to be removed for viral titer determination on day 3 (72 hours post infection). Viral titer will be determined by real-time PCR in the serum, spleen, liver, small intestine, and kidney. With the exception of the spleen, viral load in these organs is increasing at 72hr post-infection in AG129 mice. The S221 viral isolate of the D2510 strain will be administered to mice (2 x 10⁶ pfu) by intravenous tail vein injection. The PMOplus and PPMO compounds will be administered at -4hrs, +20hrs, 2, 4, 6 and 8 days after infection by the intraperitoneal route.

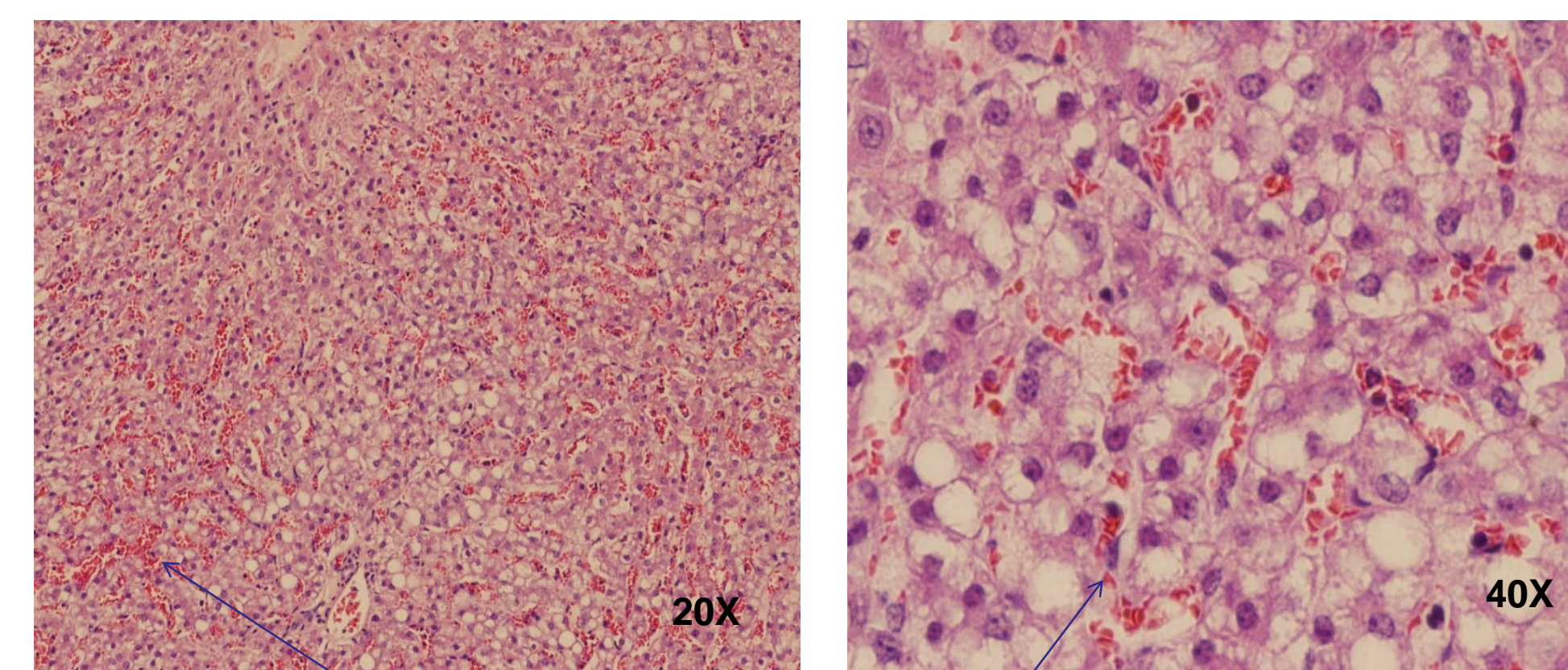
Oligomers.

PMO	5'SL:	5'-CGG TCC ACG TAG ACT AAC AAC T-3'
PMO	3'CS:	5'-TCC CAG GCT CAA TAT GCT GTT T-3'
PPMO-P007	5'SL:	5'-CGG TCC ACG TAG ACT AAC AAC T-(RXR) _n
PPMO-P007	3'CS:	5'-TCC C+AG CG+T CAA +TAT GCT GTT T-(RXR) _n
PPMO-B	5'SL:	5'-CGG TCC ACG TAG ACT AAC AAC T-(RXRRBR) _n
PPMO-B	3'CS:	5'-TCC C+AG CG+T CAA +TAT GCT GTT T-(RXRRBR) _n
PMOplus	5'SL:	5'-CGG TCC +ACG +TAG AC+T AAC AAC T-3'
PMOplus	3'CS:	5'-TCC C+AG CG+T CAA +TAT GCT GTT T-3'
PPMO	Scr:	5'-GCC TAG GAT CCA CGG TGC GC-(RXRRBR) _n
PMOplus	Scr:	5'-GCC TAG GAT CC+A CCG +TGC GC-3'



Ferret Model of Dengue 2 Infection:

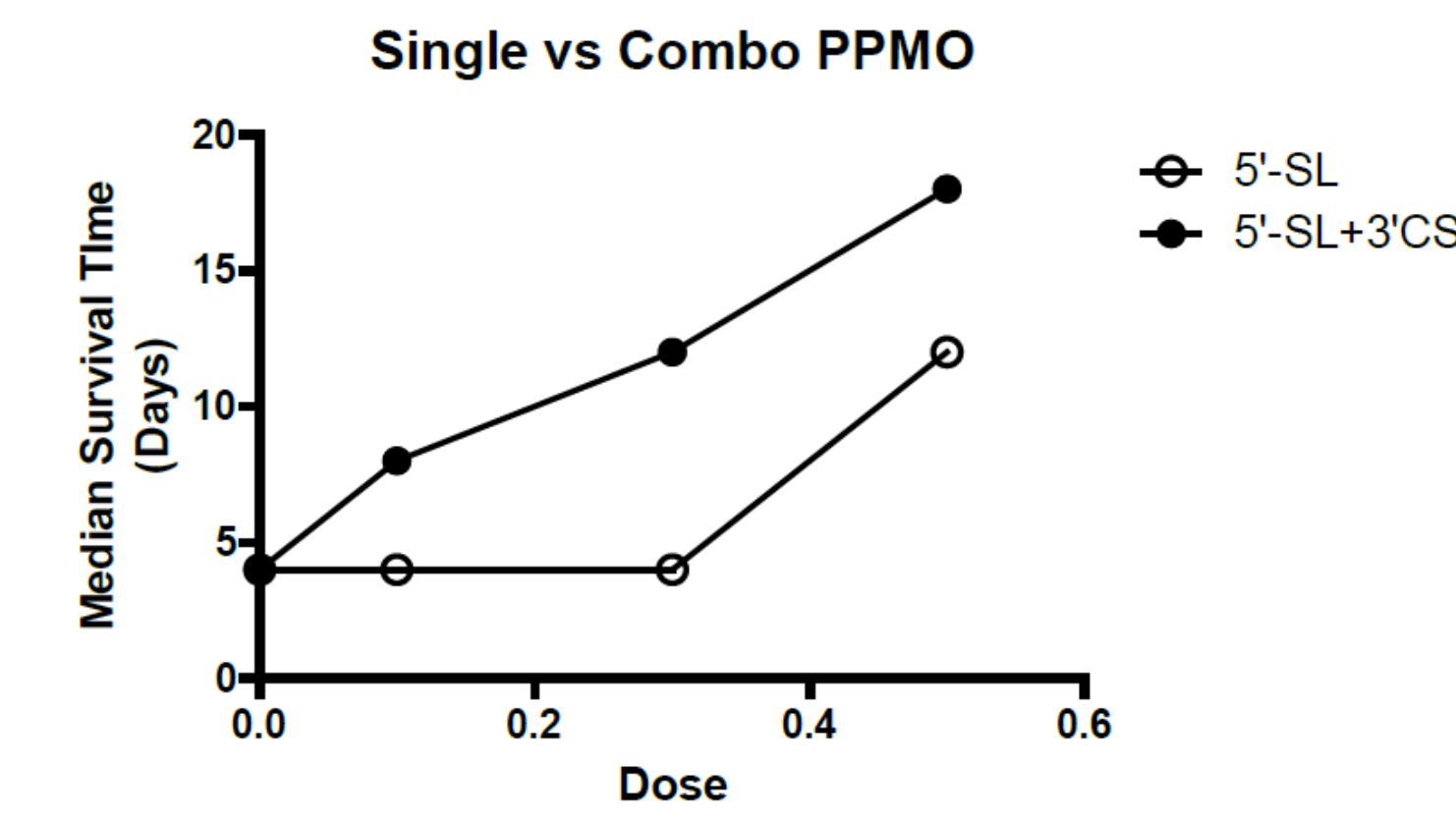
Dengue Induced Viral Pathology: Day 1 Liver (10⁶pfu/ml)



Hemorrhage

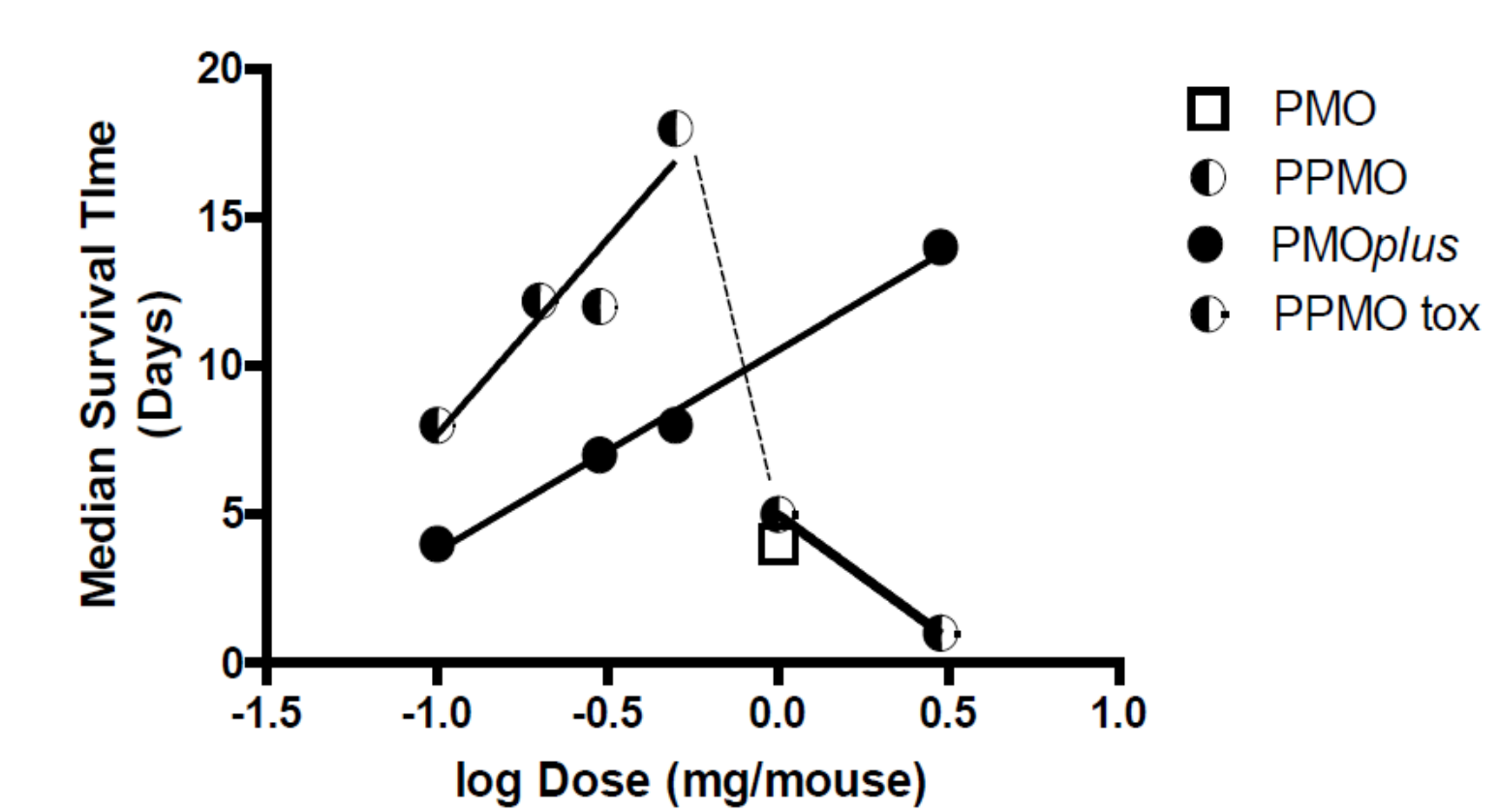
RESULTS:

FIGURE 1. Single Target Versus Dual Targeting Strategy



The observations indicate that with the same total dose, a combination of both the 5'-SL and 3'-CS are more effective. A lower total dose limits exposure and subsequently potential toxicities. We conclude the combined agent is more effective in the mouse model.

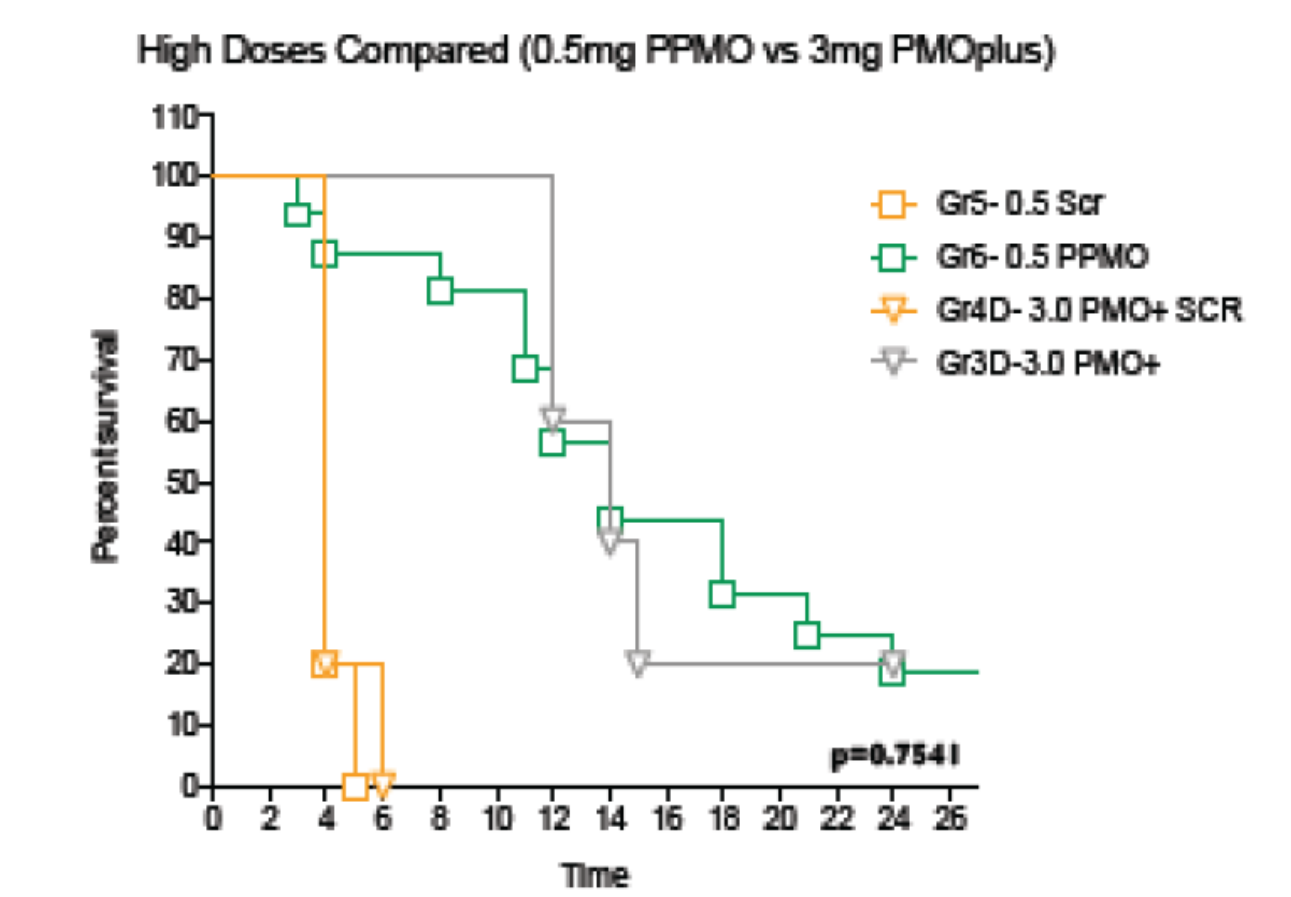
FIGURE 3. Comparison of PMO Chemistry Variations



The PMOplus is approximately 1 log more potent than a PMO but does not cause adverse events at 3.0 mg/mouse as indicated in Figure 3. We conclude the PMOplus is the optimal chemistry type because it is effective and has an expected greater therapeutic index. This broader safety profile is sought for further development given the unpredictable influence infection from Dengue may introduce to the prospective patients.

I. MOUSE EFFICACY STUDIES

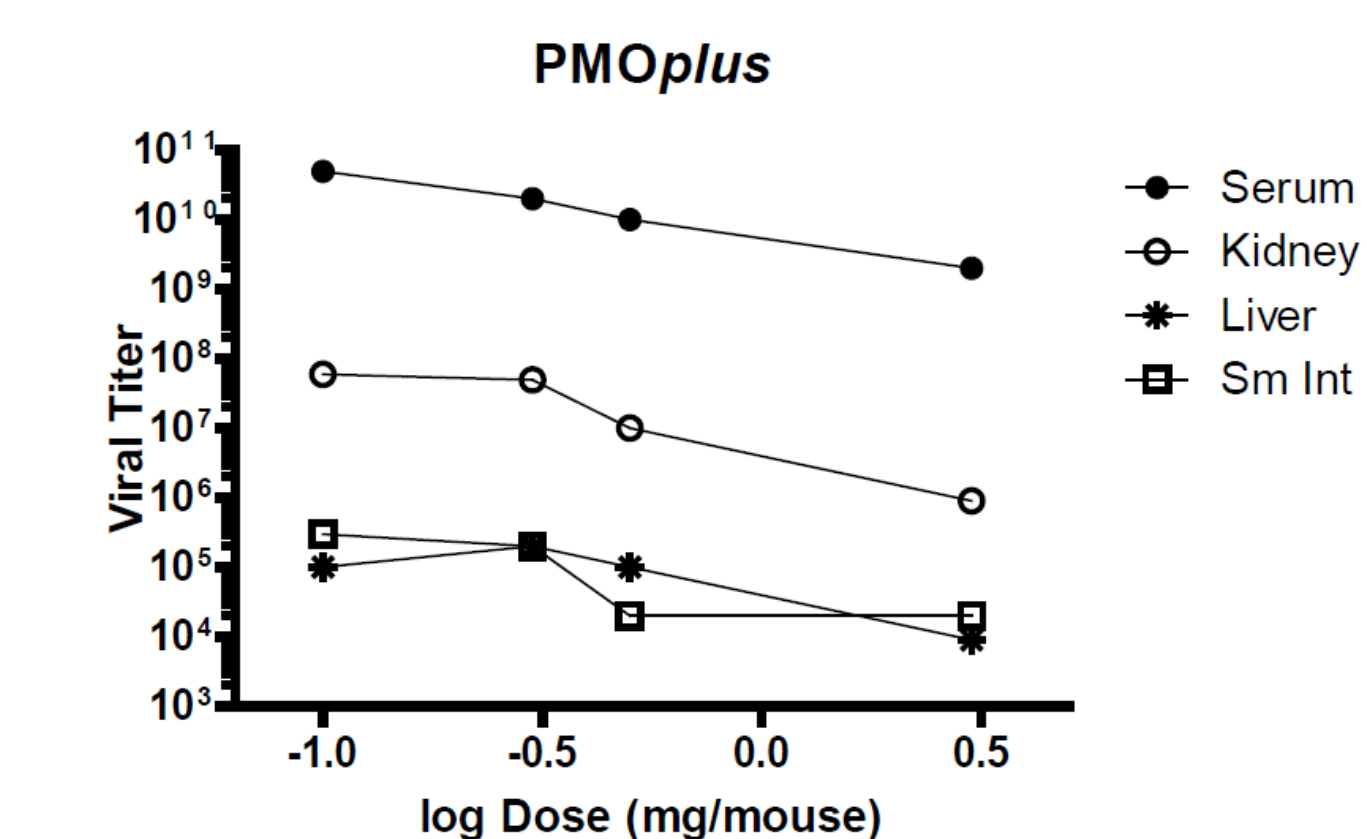
Figure 2. Survival Curves for PPMO and PMOplus



No statistical difference between 0.5 PPMO (n=16) and 3.0 PMOplus (n=5)

The PMOplus at 3mg/mouse provided equivalent long term survival as 0.5mg/mouse of PPMO. Both PPMO and PMOplus demonstrate excellent sequence-specificity.

FIGURE 4. Viral Titer Reduction

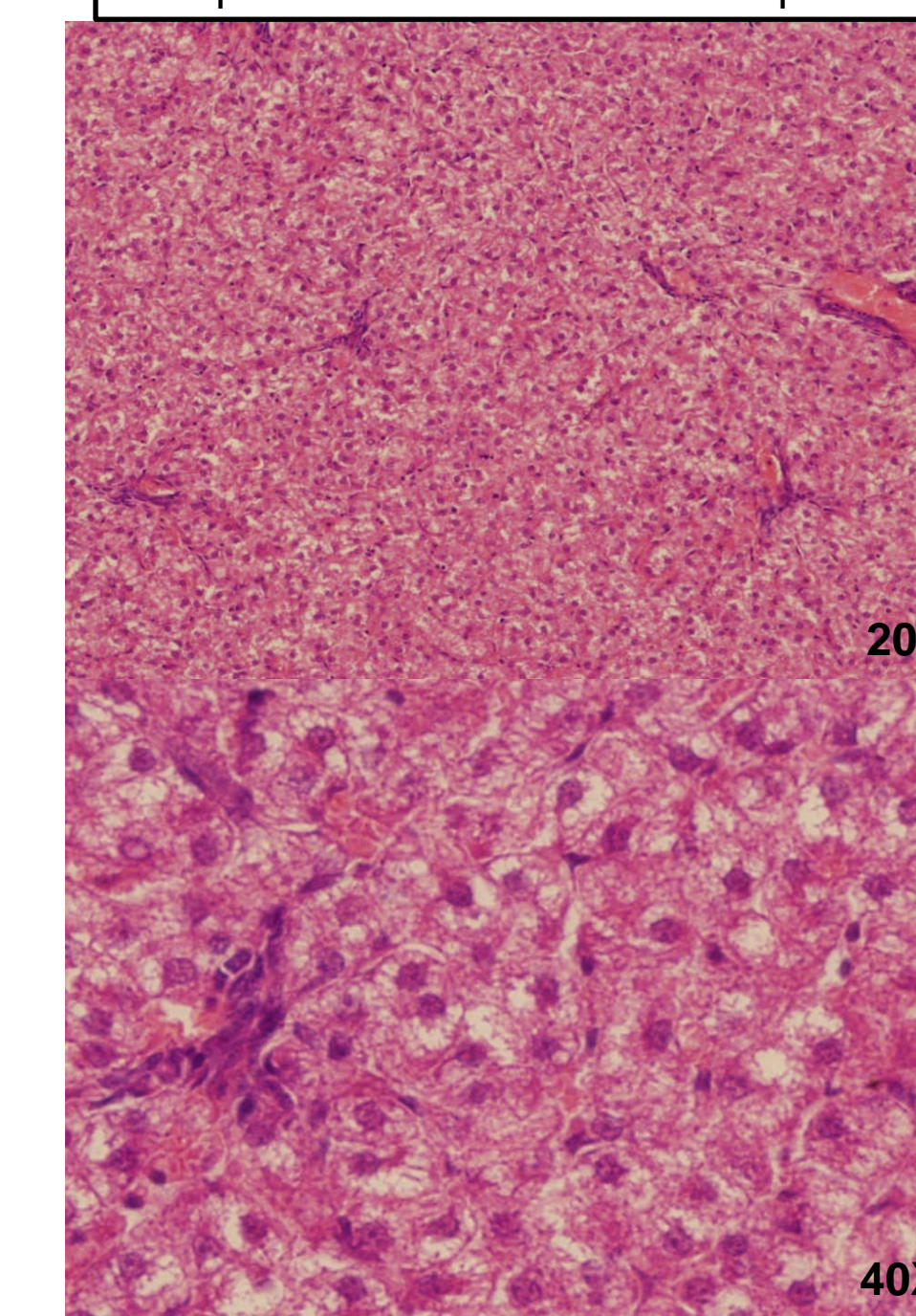


The PMOplus combination agents provide for a linear relationship between dose and reduction in viral titer in serum, liver, kidney and small intestine (Figure 4). In each case the relationship between dose and magnitude of viral titer reduction is similar, suggestive of the fact the PMOplus agents effectively distribute to each of these tissues.

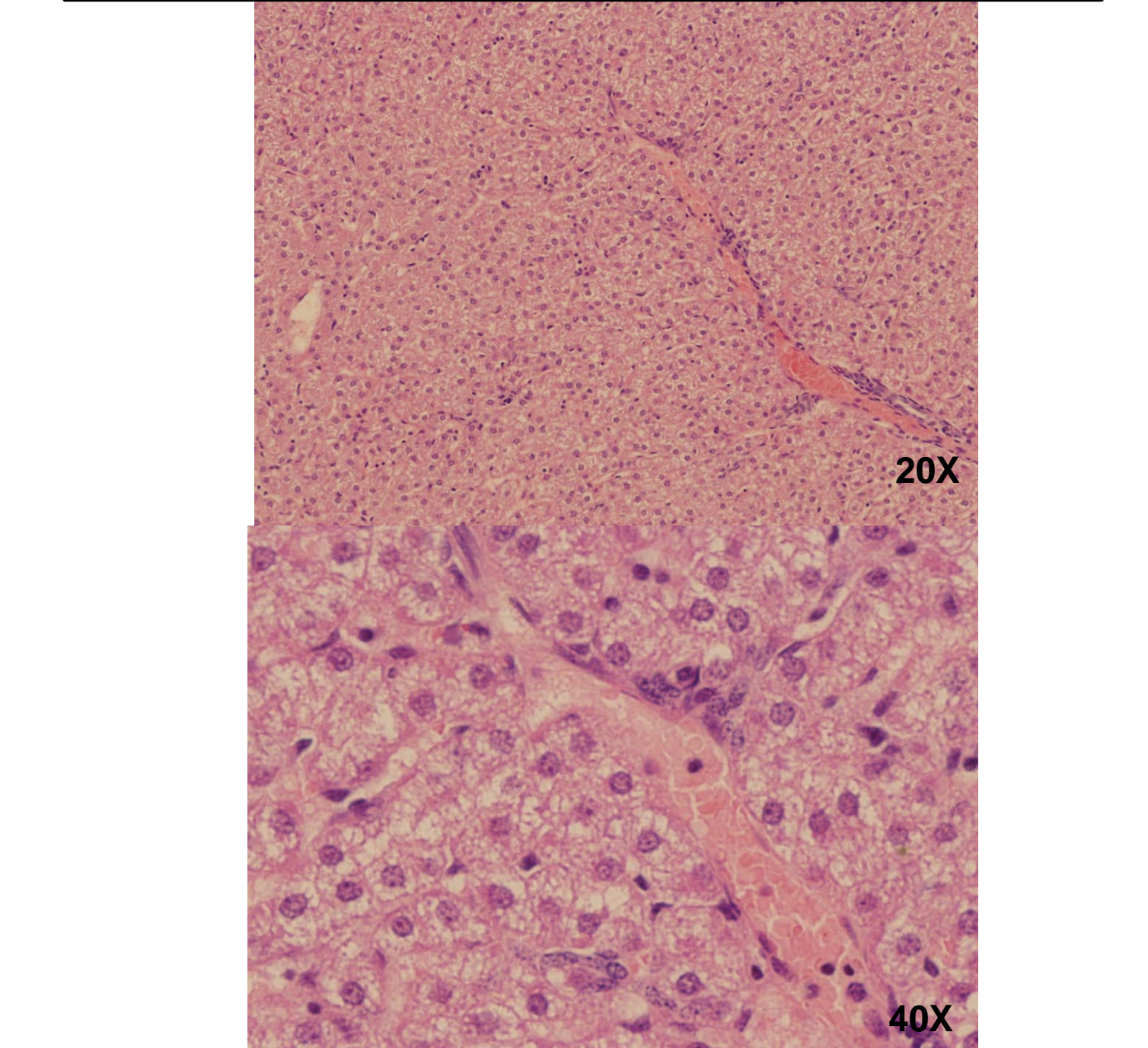
II. FERRET EFFICACY STUDIES

Group	Virus	Treatment	Number of Ferrets	Endpoints
1	DV2 10 ⁵ pfu/ml	PBS	8	Histology Plasma Titer Tissue Titer
2	DV2 10 ⁶ pfu/ml	PBS	8	
3	DV2 10 ⁵ pfu/ml	150 mg/kg i.p.	8	
4	DV2 10 ⁶ pfu/ml	150 mg/kg i.p.	8	

Group 2: No Treatment DV2 10⁶ pfu/ml



Group 4: Treatment 5'-SL + 3'-CS (150mg/kg) DV2 10⁶ pfu/ml



Conclusions:

1. A combination of the 5'-SL and 3'-CS (AVI-6006) are the optimal for establishing an antiviral for Dengue from studies in the mouse model.
2. The PMOplus chemical version of the PMO represents a balance between enhanced potency and broad therapeutic index.
3. Four or fewer cationic linkages in a PMOplus compound are non-toxic up at doses up to 100 mg/kg administered daily for four days.
4. AVI-6006 reduces viral titer in plasma and tissues in a dose dependent manner. Reduction in viral titer is positively correlated with survival in the mouse model.
5. The ferret represents a new and meaningful model for evaluation of Dengue Viral infection
6. AVI-6006 reduced the liver pathology induced by Dengue 2 in the ferret model.