Toxicology evaluation of TT-034 demonstrates durable expression in hepatic tissues without long term adverse effects on endogenous miRNA levels

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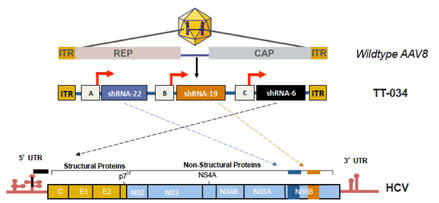
Abstract

Background: Because the HCV viral genome is comprised of a single strand of RNA and its replication occurs strictly within the cytoplasm, it is an ideal candidate for therapeutics based upon RNA interference (RNAi). TT-034 is a novel anti-viral agent based on RNAi. It is currently in phase I/IIa clinical studies for the treatment of chronic Hepatitis C Virus infection. TT-034 uses the process of DNA directed RNAi interference (ddRNAi) which triggers the cell's own transcriptional machinery to continuously produce steady state levels three independent short hairpin RNA (shRNA) to target 3 independent regions of the HCV genome. Designed to be administered as a single treatment delivered intravenously, TT-034 uses an Adeno-Associated Virus type 8 (AAV8) capsid to deliver a recombinant genome preferentially into hepatocytes.

Results: Biodistribution analyses presented here demonstrate that 90% of the vector distributes into liver tissues, with close to 100% transduction of the liver hepatocytes, as assessed by in situ hybridization. Furthermore, the durability to expression following a single injection, as assessed by qPCR, demonstrated that shRNA expression persisted for the duration of the 180 day experiment. Because previous reports have suggested that high expression of shRNA may cause global dysregulation of endogenous miRNA processes within cells, the impact of long term expression of TT-034 on endogenous miRNA levels was interrogated. Analyses were performed using RNA isolated from liver biopsies at Day 15, and from liver and heart tissues collected 60 or 180 days post TT-034 administration. This miRNA profiling demonstrated reliable detection of 260 microRNAs in the primate heart samples, as compared to 266 in liver and 269 in the liver biopsy samples. The analyses of heart tissues demonstrated that there was no statistical difference across the groups treated with TT-034 (ANOVA Benjamini Hochberg (BH) pvalue < 0.05). Although the liver biopsy samples showed significant effects in 27 microRNA, analyses of the day 60 and day 180 liver samples showed no statistical differences from the control group.

Conclusions: Collectively, these data suggest that TT-034 is not likely to have any adverse effects in miRNA processes in primate cells.

Expression of Three anti-HCV shRNA From a Recombinant AAV Expression Cassette

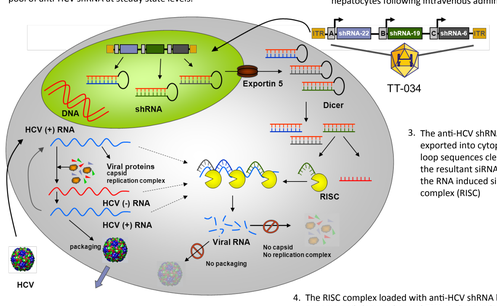


- TT-034 is delivered via a single intravenous infusion, representing the sole treatment
- 3 independently transcribed shRNA elements target 3 separate, well-conserved regions of the HCV genome; helps prevent the generation of viral escape mutants
- Delivery uses capsid derived from adeno associated virus (AAV), a non-integrating, non-pathogenic virus used in over 117 clinical trials
- Sustained expression (potentially years based on other clinical studies using Factor IX) following a single injection
- Complete transduction of liver hepatocytes with serotype 8 (AAV8)

TT-034 Mechanism of Action – RNA Interference

2. TT-034 uses the cell's own transcriptional machinery to produce a constantly replenishing pool of anti-HCV shRNA at steady state levels.

1. TT-034 is comprised of an AAV8 capsid that delivers a non-integrating recombinant genome to hepatocytes following intravenous administration.

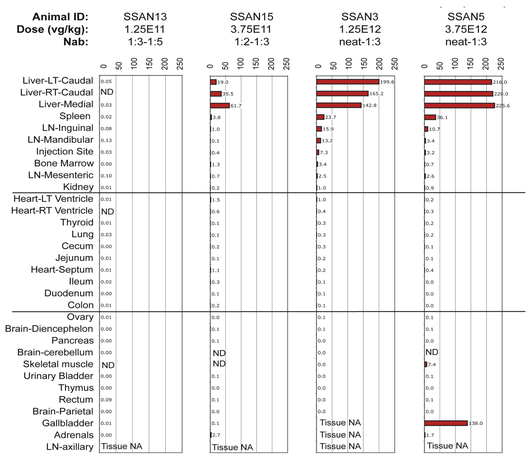


3. The anti-HCV shRNAs are exported into cytoplasm, the loop sequences cleaved and the resultant siRNAs enter the RNA induced silencing complex (RISC)

4. The RISC complex loaded with anti-HCV shRNA binds to the HCV genome and cleaves the viral RNA at three independent, well conserved regions rendering the RNA genome incapable of being packaged into nascent virions or serving as a template for the translation of viral proteins.

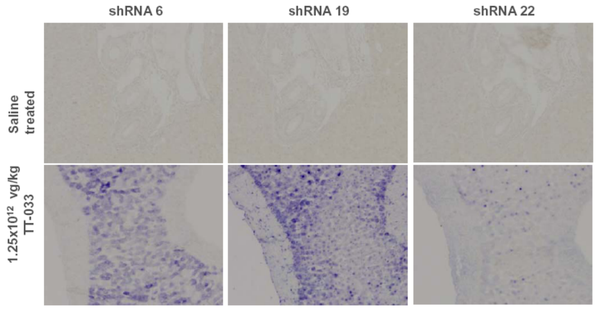
5. Because the shRNA is produced continuously for months or even years following the single administration, the anti-shRNA produced from TT-034 may be able to protect the transduced hepatocytes from re-establishment of HCV infection long term.

Biodistribution of AAV8 in Cynomolgus Monkeys



- 4 different cynomolgus monkeys were administered with increasing doses of vector. After 50 days, the animals were sacrificed, their tissues harvested and analyzed for the presence of the recombinant DNA construct

AAV Permits Efficient Hepatocyte Transduction Following IV Administration of a Clinically Relevant Dose



- Cynomolgus monkeys were administered 1.25e12 vg/kg of the shRNA expressing vector by intravenous injection or were treated with saline. Hepatic tissues were harvested 30 days later.
- Qualitative *In situ* hybridization (ISH) analyses reveals near complete transduction of hepatocytes.

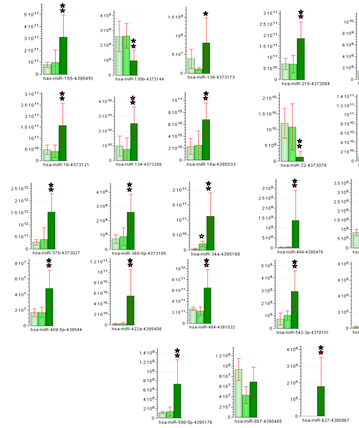
Minimal Changes in endoenous miRNA Levels Occur Within Liver Tissues within Two Weeks of Dosing

- Alterations in endogenous microRNAs following the administration of TT-034 were evaluated at 60 and 180 days post injection in heart and liver samples. Additionally, liver biopsies taken at Day 15 from these same animals were also analyzed for microRNA expression. To control for variability in RNA loading, the expression values in a sample were normalized to the geometric mean of the least variable endogenous reference microRNA across the sample set.

microRNA assay	Control vs Mid Dose BH Q-Value	Control vs High Dose BH Q-Value	Mid Dose vs High Dose BH Q-Value	One-Way ANOVA BH Q-Value
has-miR-155-4395459	0.9582191	0.002829842	0.011876984	0.028793612
hsa-miR-130b-4373144	0.9582191	0.014244441	0.011482662	0.032619722
hsa-miR-134-4373299	0.8214493	0.014244441	0.01626282	0.032619722
hsa-miR-136-4373173	0.11172516	0.20635918	0.01026292	0.047261156
hsa-miR-16-4373121	0.8778927	0.034932047	0.018300978	0.046851873
hsa-miR-18a-4395533	0.9582191	0.005900402	0.010584564	0.046185166
hsa-miR-18b-4395328	0.9412786	0.005900402	0.007907171	0.029738517
hsa-miR-20b-4373263	0.920716	0.018142032	0.011874954	0.046851873
hsa-miR-212-4373087	0.8214493	0.000988938	0.003777842	0.001097821
hsa-miR-215-4373084	0.9582191	0.006494548	0.008700965	0.03487566
hsa-miR-22-4373079	0.8778927	0.016303819	0.01889258	0.023056291
hsa-miR-26b-4395167	0.8214493	0.01587737	0.021307722	0.046851873
hsa-miR-34a-4395168	0.036287441	0.000190729	0.007907171	0.001097821
hsa-miR-369-5p-4373195	0.9412786	0.003556559	0.009348501	0.03487566
hsa-miR-375-4373027	0.9582191	0.001392706	0.01026292	0.020548942
hsa-miR-409-5p-4395442	0.9582191	0.010273104	0.01026292	0.040839236
hsa-miR-422a-4395408	0.9582191	0.004620023	0.007907171	0.014084894
hsa-miR-484-4381032	0.49100506	0.035226353	0.013886789	0.046851873
hsa-miR-499-4395469	0.140727	0.000190729	0.053377842	0.013116413
hsa-miR-491-5p-4381053	0.8214493	0.012924163	0.021201352	0.046851873
hsa-miR-494-4395476	0.9412786	0.002865206	0.007907171	0.01342723
hsa-miR-542-3p-4378101	0.606358106	0.025607601	0.01026292	0.032619722
hsa-miR-582-3p-4395510	0.33835694	0.002426942	0.007907171	0.00725529
hsa-miR-590-5p-4395176	0.9582191	0.019160036	0.020423278	0.044500478
hsa-miR-618-4380996	0.11172516	0.000988938	0.007907171	0.003419039
hsa-miR-627-4380967	NaN	NaN	0.007907171	0.004931247
hsa-miR-887-4395485	0.024492491	0.12403425	0.0799654	0.046851873

*Red denotes up and green denotes down regulation.

- Shown to the right, a comparative analysis (Benjamini-Hochberg FDR value) reveals changes in 27 microRNAs that were significantly different between the control group, mid dose (1e12 vg/kg), and high dose (1e13 vg/kg) animals in Day 15 biopsy samples. The q-values and ANOVA results are shown in the above table.
- Although previous reports (*Nature* **441**, 537-541) suggest that overexpression of shRNA may cause global dysregulation of endogenous miRNA pathways and corresponding downregulation of mature miRNA levels, most changes were noted to occur in the high dose group as a result of up regulation.
- It has not been determined if the changes noted in the liver biopsies are as a result of specific shRNA expression or are temporarily alterations as a result of large levels of liver transduction by the recombinant viral vector.



Yet, No Long Term Impact of TT-034 on Endogenous miRNA Levels in Cardiac and Liver Tissues

Eigenrow 1: 27.9%

Heart Analyses	Heart 60 Control	Heart 60 Mid Dose	Heart 60 High Dose	Heart 180 Control	Heart 180 Mid Dose	Heart 180 High Dose
Heart 60 Control	0.9827	0.9754	0.9782	0.9758	0.9802	0.9794
Heart 60 Mid Dose	0.9754	0.9787	0.9790	0.9706	0.9822	0.9803
Heart 60 High Dose	0.9782	0.9790	0.9800	0.9757	0.9826	0.9821
Heart 180 Control	0.9758	0.9706	0.9757	0.9746	0.9761	0.9760
Heart 180 Mid Dose	0.9802	0.9822	0.9826	0.9761	0.9860	0.9852
Heart 180 High Dose	0.9794	0.9803	0.9821	0.9760	0.9852	0.9845

Scale: 0.84, 0.86, 0.88, 0.9, 0.92, 0.94, 0.96, 0.98, 1

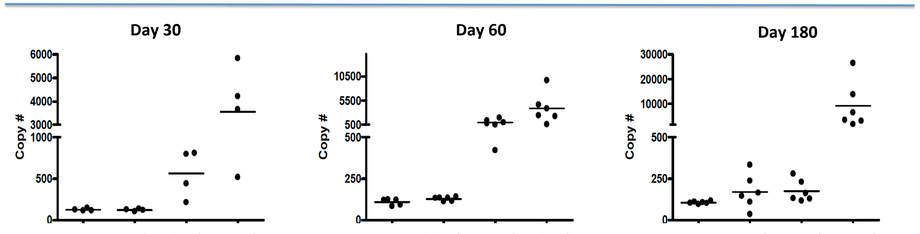
Eigenrow 1: 58.9%

Liver Analyses	Liver 60 Control	Liver 60 Mid Dose	Liver 60 High Dose	Liver 180 Control	Liver 180 Mid Dose	Liver 180 High Dose	Liver TC Control	Liver TC Mid Dose	Liver TC High Dose
Liver 60 Control	0.9749	0.9784	0.9725	0.9563	0.9731	0.9577	0.8743	0.8773	0.8672
Liver 60 Mid Dose	0.9784	0.9856	0.9754	0.9519	0.9733	0.9561	0.8802	0.8804	0.8648
Liver 60 High Dose	0.9725	0.9754	0.9727	0.9591	0.9740	0.9575	0.8679	0.8743	0.8737
Liver 180 Control	0.9563	0.9519	0.9591	0.9622	0.9605	0.9587	0.8443	0.8577	0.8796
Liver 180 Mid Dose	0.9731	0.9733	0.9740	0.9605	0.9765	0.9607	0.8565	0.8660	0.8703
Liver 180 High Dose	0.9577	0.9561	0.9575	0.9587	0.9607	0.9518	0.8524	0.8627	0.8769
Liver TC Control	0.8743	0.8802	0.8679	0.8443	0.8565	0.8524	0.9755	0.9572	0.8969
Liver TC Mid Dose	0.8773	0.8804	0.8743	0.8577	0.8660	0.8627	0.9572	0.9447	0.9093
Liver TC High Dose	0.8672	0.8648	0.8737	0.8796	0.8703	0.8769	0.8969	0.9093	0.9524

Scale: 0.84, 0.86, 0.88, 0.9, 0.92, 0.94, 0.96, 0.98, 1

- The correlation table from Principal Component Analysis shows no significant differences in 260 miRNA quantified in TT-34 treated heart samples analyzed at different time points relative to control. Statistical differences were analyzed by ANOVA Benjamini Hochberg (BH)with a p-value < 0.05.
- The correlation table of a Principal Component shows significant differences in 269 miRNA quantified in TT-034 treated liver biopsy (True Cut, TC) samples taken at day 15 versus 266 miRNA detected in control animals. Yet, liver samples analyzed at day 60 and 180 from the mid or high-dose groups are not significantly different from their respective controls.

Long Term Expression Expression of shRNA-22 in Liver Following a Single Dose of TT-034



- Cynomolgus monkeys were administered with 1e11, 1e12 or 1e13 vg/kg TT-034. After 50 days, the animals were sacrificed, their tissues harvested and analyzed for the presence of each of the anti-HCV shRNAs

Summary

- Biodistribution analysis of TT-034 delivered intravenously reveals that > 90% of administered vector that is quantified is detected in hepatic tissues
- Clinically relevant doses (1.25e12 vg/kg) can result in complete liver transduction in non human primates
- Intravenous administration of TT-034 results in dose-dependent and durable expression of the three anti-HCV shRNA in the liver of cynomolgus monkeys.
- In the liver, the highest dose induced measureable, but minimal, alterations in the expression level of endogenous miRNAs in the day 15 liver biopsy samples.
- It is not clear if the changes are a result of shRNA expression or the transduction of hepatocytes with AAV vectors, post vector administration.
- The alterations in endogenous miRNA levels, in general, did not persist in the Day 60 or Day 180 samples indicating that global miRNA expression is not dysregulated long term.
- No alterations in endogenous miRNA expression were detected in the heart.