Long-acting tenofovir and nitazoxanide prodrug formulations suppress HBV replication

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Abstract

Background: To eliminate HBV infection complete suppression of progeny virus is required for final elimination of infected cells. We posit that enhancement of mandated innate and adaptive immune responses, against HBV, can be achieved with antiviral therapy alone. To this end, we now offer a new means to achieve such viral suppression by enhancing the potency of *nitazoxanide (NTZ),* a broad-spectrum antiviral and immune stimulating agent and *tenofovir* (TFV), a nucleoside reverse transcriptase inhibitor. This drug combination was transformed into hydrophobic prodrug nanocrystals then stabilized into aqueous nanosuspensions. The modifications increase the half-lives of both drugs, enhanced the drug's efficacy and improved its distribution to liver cells where HBV resides.
Methods: NTZ and TFV prodrugs (M1NTZ and M1TFV) were synthesized then nanoformulated creating NM1NTZ and NM1TFV. Drug uptake and retention was determined in human monocyte-derived macrophages (MDM). The HBV-producing human hepatocellular carcinoma HepG2.2.15 cell and humanized liver TK-NOG mice were used to test antiviral activities. HBV infected mice received a single 75 mg/kg intramuscular combination injection of NM1NTZ and NM1TFV. HBV DNA, cccDNA and HBe/sAg were monitored for 48 h and for 10 weeks in cells and infected animals.
Results: NM1NTZ and NM1TFV were 250-350 nm in size, held a polydispersity index of <0.2. The drug loading capacity was > 70%. NM1TFV was readily taken in MDM with sustained prodrug levels of up to 30 days. TAF, in contrast, was hydrolyzed within one day. HBV DNA release by (NM1TFV) and cccDNA pools (NM1NTZ and NM1TFV) were easily seen. The combination long acting prodrugs reduced HBV DNA in the plasma of humanized mice. The levels reached undetectable levels in 2/4 animals at four weeks. Human cells were readily detected (**Fig. 3**). The remaining two animals showed > log decrease in plasma viral load at equivalent times. Animals were monitored for 10 weeks.
Conclusion: Long-acting TFV and NTZ prodrugs sustained antiviral activity in humanized mice for a month. This followed a single dose. These data sets support monthly dosing of NM1TFV and NM1NTZ for HBV infections.

NTZ Prodrug Characterization

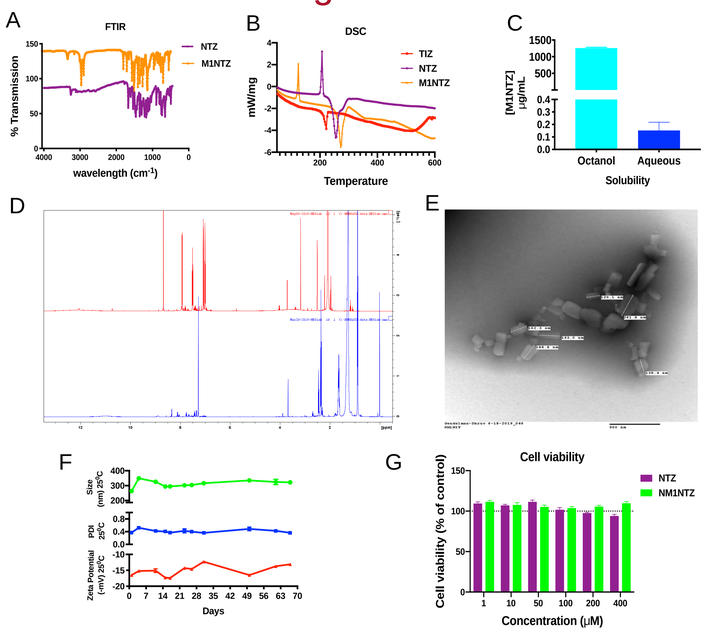


Figure 1: (A) FTIR spectra of M1NTZ and NTZ exhibiting absorption bands at 2915 cm^{-1} and 2850 cm^{-1} in the prodrug spectrum to confirm native drug modification (B) DSC spectra of M1NTZ, NTZ, and parent tizoxanide (TIZ) demonstrated differences in the melting points for each compound (C) Aqueous and octanol solubility of M1NTZ at room temperature demonstrates enhanced hydrophobicity and lipophilicity (D) ^{1}H NMR spectra of NTZ (red) and M1NTZ (blue) confirmed chemical modification of the parent drug (E) Nanocrystal morphologies were evaluated by TEM (F) Determination of nanocrystal stability by measurement of particle size, PDI and zeta potential over 70 days (G) Evaluation of mitochondrial function in macrophages.

TFV Prodrug Characterization

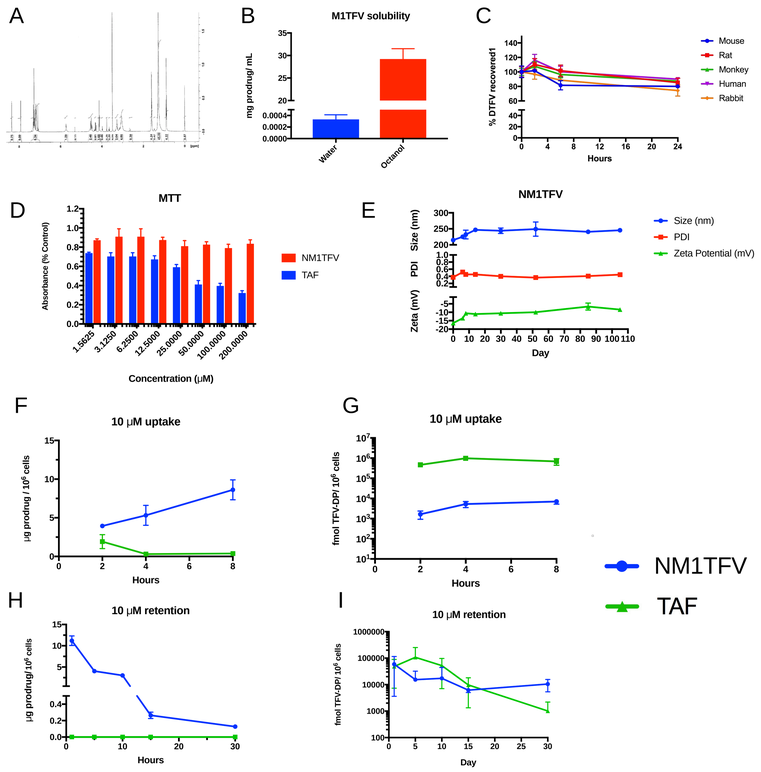


Figure 2: (A) Proton NMR spectrum of M1TFV confirmed the chemical structure of the prodrug (B) Modification of TFV enhanced hydrophobicity and lipophilicity of M1TFV. (C) Stability of M1TFV in plasma of mouse, rat, monkey, human, and rabbit plasma over the course of 24hrs at 37°C. Previous studies have shown that *in vivo* accumulation of nucleoside active metabolites inside cells could be facilitated by prodrugs that exhibit greater stability in plasma. (D) Evaluation of mitochondrial activity demonstrated that NM1TAF exerted no adverse effects to cell viability at 200 µM of drug or less. (E) Physicochemical parameters (size, PDI and zeta potential) of NM1TFV nanocrystals were monitored by dynamic light scattering. (F and G) Time course concentration of prodrug and TFV-DP following drug uptake in MDM. For cell uptake, MDM were treated with 10 µM TAF or NM1TFV for 1-8 h. (H and I) Prodrug and TFV-DP retention in MDM was determined after an 8 h drug loading followed by half media exchanges every other day for 30 days. Data are expressed as mean ± SD for n = 3 samples/group.

Conclusions

NM1NTZ and NM1TFV had average particle sizes of 250-350 nm, polydispersity index of <0.2 and drug loading capacity of > 70%. NM1TFV formulation was taken up by MDM with sustained drug levels for 30 days; whereas native drugs were metabolized within one day. The combination long acting prodrug therapy reduced HBV DNA in plasma of humanized mice to undetectable levels in 2/4 animals tested at four weeks with readily detected human cells (**Fig 3**). Long-acting TFV and NTZ prodrugs sustained antiviral activity in humanized mice for a month after a single dose. These data sets support the potential of monthly NM1TAF and NM1NTZ dosing for treatment of HBV infections.

Pharmacodynamics

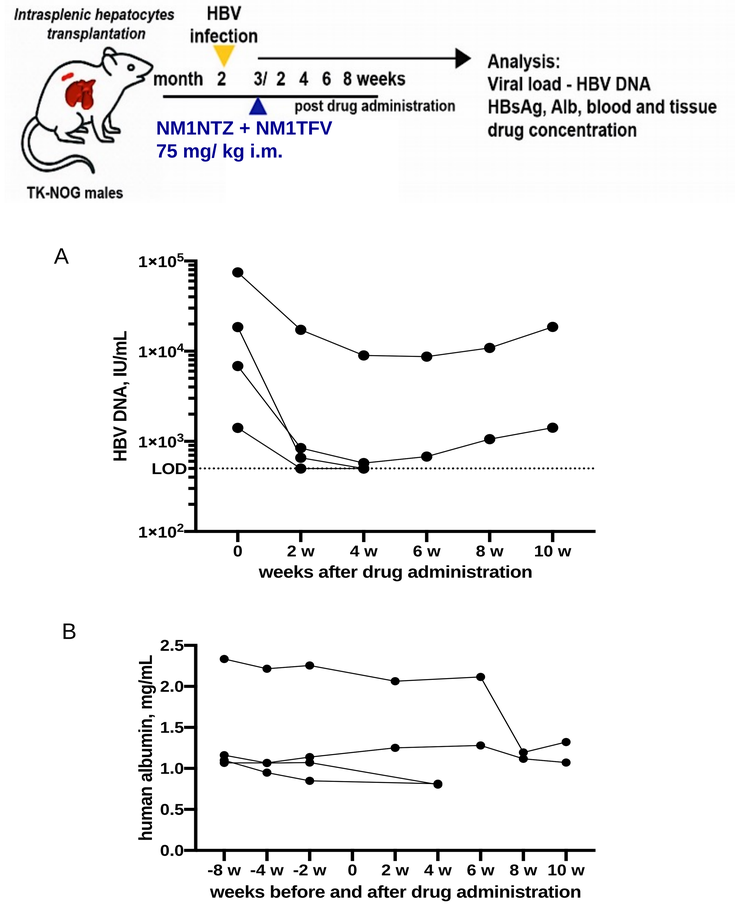


Figure 3: Evaluation of NM1TFV and NM1NTZ combination regimen in HBV infected humanized liver TK-NOG mice. Animals were infected with 10^6 GE HBV and 2-months post infection; a single intramuscular injection of the combination therapy was administered at 75 mg/kg of native drug equivalents for each prodrug formulation. HBV DNA, cccDNA and HBe/sAg were monitored for 48 h and for 10 weeks in cells and animals, respectively. Reduction of HBV DNA in two animals below limit of detection (LOD) was found. These animals were euthanized to measure liver drug concentrations. * P values obtained by t-test. (B) Human albumin levels measured over 6 weeks.

Acknowledgement

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