Metabolically Stable ω-Functionalized Lipid Prodrugs of Tenofovir Have the Potential to Serve as Safer, Long-Acting Antiretrovirals for the Treatment of HIV.

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Background: Tenofovir exalidex (TXL), a potent, plasma stable and orally bioavailable lysophospholipid-derived prodrug of tenofovir (TFV), was designed to target natural lipid uptake pathways and an intracellular cleavage mechanism by phospholipase C (PLC) to deliver TFV to HIV infected cells. However, like tenofovir disoproxil fumarate (TDF) and tenofovir alafenamide (TAF), the utility of TXL is limited due to considerable metabolism by the liver, namely by CYP450-mediated ω-oxidation at the terminus of the lipid prodrug. Undesired metabolism by the liver not only compromises the amount of prodrug available to access HIV infected cells but also, due to chronic use, increases the potential for toxicities which can negatively impact adherence to treatment. Herein, a series of TXL analogues were designed and synthesized which feature metabolically stable motifs at the terminus of the lipid chain in order to prevent undesired ω-oxidation, mitigate toxicity and deliver larger fractions of prodrug, intact, into systemic circulation and, ultimately, to HIV infected cells.

Methods: The *in vitro* assessment of cellular toxicity and HIV-antiviral activity of all TXL analogues were carried out in HEK293T cells. Anti-HIV activity was evaluated in an *in vitro* single-cycle non-replicating HIV pseudoviral assay which relied on the expression of luciferase to assess inhibition of viral reverse transcriptase. The *in vitro* metabolic stability of all TXL analogues was assessed by incubating compounds in human liver microsomes (HLMs). Resulting levels of prodrug were then quantified at five time points between 0 and 2 hours by LC-MS/MS. Three TXL analogues with the most promising *in vitro* properties were then assessed in an *in vivo* mouse plasma and liver pharmacokinetic (PK) experiment. Male C57BL/6 mice were dosed with a single 10 mg/kg oral dose of TXL analogue using a 90:10 olive oil:ethanol solution as a vehicle. Levels of prodrug and TFV were then quantified at various time points in plasma and in the liver using LC-MS/MS.

Results: Overall, TXL analogues featuring terminally substituted lipid prodrugs demonstrated superior *in vitro* metabolic stabilities ($t_{1/2} \geq 2$ hours) in HLMs relative to TXL ($t_{1/2} = 42$ minutes). Moreover, TXL analogues terminally substituted with trimethylsilyl (TMS) acetylene, trifluoromethyl (CF_3) acetylene and CF_3 motifs exhibited nanomolar potency in the whole cell HIV assay with IC_{50} values of 69 nM, 23 nM and 49 nM respectively. The promising *in vitro* properties of these three TXL analogues warranted further evaluation in a mouse PK study. In general, the three TXL analogues demonstrated enhanced systemic exposure levels in plasma

relative to TXL, as indicated by the $AUC_{0\text{-}24h}$. The CF_3 analogue exhibited the highest plasma $t_{1/2}$ among the tested TXL analogues. In the plasma and the liver, relative levels of prodrug to the metabolite TFV, represented by the ratio of prodrug $AUC_{0\text{-}24h}$ / TFV $AUC_{0\text{-}24h}$, were far superior for the TMS acetylene and CF_3 analogues relative to TXL, which was readily metabolized to TFV.

Conclusions: The *in vitro* and *in vivo* results herein serve as a proof-of-concept that supports the hypothesis that introducing metabolically stable motifs at the terminus of lipid prodrugs of TFV would mitigate undesired metabolism by the liver. Furthermore, the observed enhanced systemic exposure levels of these terminally substituted TXL analogues and their relatively superior stability in mouse plasma and liver indicate that these prodrugs have the potential to act as safer and long-acting therapeutics for the treatment of HIV.