Investigation of the anti-HBV Activity of TL020 and the Effects on Host Protein Expression

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Background

Worldwide, about 260 million people are chronically infected by the hepatitis B virus (HBV), which claims around 900,000 lives per year. The current drug treatments are with pegylated interferon, which rarely achieves viral clearance and can have severe side-effects, or with nucleos(t)ide analogues which are lifelong management programs that do not clear the infection[1]. In an in-vitro screen using HepG2.2.15 cells, the compound TL020 was shown to inhibit HBV replication. The dose for viral inhibition was 30-80 fold lower than that observed for cytotoxicity. The mechanism of action is expected to be different than those involved in the above-mentioned treatments. As such, investigation of this mechanism of action is important for development of more potent and less toxic compounds as potential clinical candidates and for subsequent drug approval. Additionally, if the mechanism of action is novel it may improve understanding of the lifecycle of HBV and help with development of new therapeutic strategies.

Introduction

The Sec61 channel is a heterotrimeric complex in the endoplasmic reticulum (ER) membrane. Approximately 40% of the human genome encodes proteins that pass through this channel during synthesis or maturation. Either an N-terminal signal peptide or a transmembrane domain target proteins to this channel and help open it to enable their translocation across the ER membrane. Despite having conserved function and structure, each signal peptide has a unique sequence and a different capacity for opening the channel. Cyclotriazadisulfonamide (CADA) is a small molecule that was shown to have anti-viral activity against human immunodeficiency virus (HIV). CADA was shown to bind to the signal peptide of human CD4, the entry receptor for HIV, block its passage through the Sec61 channel, causing its degradation and reducing its levels on the cell surface (Figure 1a)[2]. Although TL020 (Figure 1b) has high potency for down-regulation of CD4, HBV does not rely on CD4 for entry or need it for any stage of its lifecycle, and the anti-HBV assay used does not detect entry inhibitors. It is likely that the anti-HBV effect of TL020 is due to down-regulation of another target protein, perhaps a host cofactor recruited by HBV for replication.

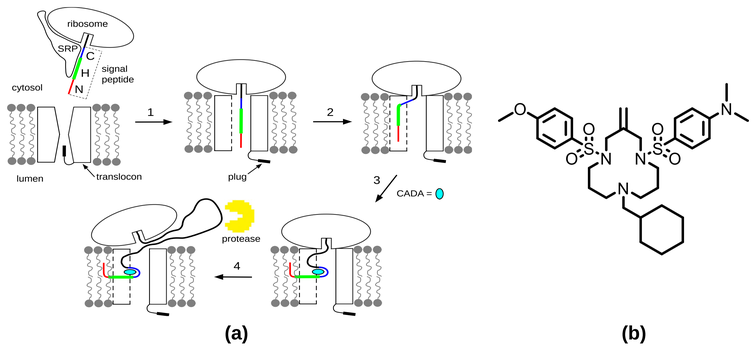

Figure 1: (a) Proposed mechanism of action for protein down-modulation by CADA and analogues. (b) TL020, a CADA analogue

Screening for anti-HBV activity

HepG2.2.15 cells are a human hepatoma cell line transfected to express HBV. This is an extremely useful model for screening for anti-HBV compounds; however, the cell line does not express the protein that acts as the entry receptor for the virus. It is only useful for finding compounds which inhibit viral protein synthesis, assembly, replication, and egress. In the primary screen the cells were incubated with concentrations from 10-0.1 µM of each compound and secreted HBV levels were quantified by collecting the cell growth media and detecting viral DNA using Northern blotting. In a secondary follow-up screen, after removing the media for testing the same as before, the levels of intracellular viral DNA were measured as well by lysing cells and performing a Northern blot. Additionally, to test for cytotoxicity a neutral red toxicity screen was performed. TL020 was found to reduce both cellular and secreted HBV DNA levels at low concentrations suggesting that it is interfering with viral protein synthesis, assembly, or replication steps of the HBV lifecycle.

Table 1: Viral DNA levels were reduced both in the media and within HepG2.2.15 cells at low concentrations of TL020. The EC_{50} and EC_{90} are the concentration that reduce viral DNA levels by 50 or 90%, respectively. The CC_{50} is the concentration required the kill 50% of cells. The SI_{50} and SI_{90} are the ratio between either EC_{50} or EC_{90}, respectively, and CC_{50}.

Assay	EC_{50} (µM)	EC_{90} (µM)	CC_{50} (µM)	SI_{50} (µM)	SI_{90} (µM)
Primary, DNA (virion)	0.49	1.2	38	78	32
Secondary, DNA (virion)	0.63	1.6	53	84	33
Secondary, DNA (cellular)	1.2	5.9	53	44	9.0

Result: Affect of TL020 on HepG2 cell protein expression

HepG2 cells are the parental cell line to HepG2.2.15 and do not express HBV. To investigate whether the effect of TL020 on HBV is due to reduction of the level of host proteins, HepG2 cells were incubated with TL020 at 20 µM or vehicle control for 2 days in serum-free media. The cells were lysed, and the proteins extracted for analyses by LC-MS/MS. There were 385 differentially expressed (DE) genes out of 4,680 genes with measurable expression. Pathways were assessed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database and the gene ontologies (GO) terms were assessed using the Gene Ontology Consortium database. KEGG Pathway analyses revealed the protein processing in the ER was significantly affected; additionally, GO analysis revealed biological processes in response to ER stress and that all five of most significantly affected cellular components pass through the Sec61 channel. This is consistent with the known mechanism of action of CADA.

Tables 2-5: Summary of changes in protein levels. DE genes compared with KEGG and GO databases.

Pathway Name	# of Genes (DE/ALL)	p-value
ECM-receptor interaction	10/25	1.1e-6
protein processing in ER	29/109	3.1e-6
bile secretion	9/17	1.8e-5
pyruvate metabolism	10/27	3.0e-5
metabolic pathways	76/605	5.4e-5
GO Term (Biological Processes)	**# of Genes (DE/All)**	**p-value**
cholesterol biosynthetic process	18/40	5.4e-10
cell redox homeostasis	13/39	8.6e-10
acute-phase response	7/14	5.5e-10
alcohol catabolic process	8/14	5.0e-5
response to ER stress	26/131	7.4e-5
GO Term (Molecular Functions)	**# of Genes (DE/All)**	**p-value**
virus receptor activity	11/28	6.5e-6
coenzyme binding	27/145	4.2e-5
lipoprotein particle receptor binding	8/21	1.6e-4
oxidoreductase activity	11/32	2.2e-4
iron ion binding	11/40	2.8e-4
GO Term (Cellular Components)	**# of Genes (DE/All)**	**p-value**
ER lumen	47/137	1.3e-24
ER membrane	67/343	1.6e-15
external side of plasma membrane	20/47	8.0e-13
integral component of membrane	116/662	8.7e-11
extracellular exosome	136/1085	4.3e-9

Result: mRNA levels and signal peptides

The mechanism of CADA compounds is a post-transcriptional prosses. Therefore, transcript levels for proteins affected by this mechanism will likely not be affected. To find proteins affected by the mechanism we focused on the 30 proteins with signal peptides and reduced expression levels. HepG2 cells were treated as before except after incubation and lysis the mRNA was extracted for analysis by qRT-PCR using SYBR green dye for quantification. Furthermore, the apparent free energy difference, $\Delta\Delta G_{app}$, for insertion of the signal peptides into the ER membrane was calculated[3]. Larger values for this calculation represent signal peptides which are thought to be more amenable to interference by small molecule inhibitors of the Sec61 mediated translocation process.

Table 6: Proteins most likely affected by the mechanism. Each is indicated by its gene name with the percent change in protein expression level according to LC-MS/MS, mRNA fold change (FC) by qRT-PCR and the *in-silico* measure of each signal peptide's ability to open the channel for protein translocation ($\Delta\Delta G_{app}$). High $\Delta\Delta G_{app}$ indicates a high energy cost to membrane insertion and channel opening.

Gene ID	% Change	mRNA FC	$\Delta\Delta G_{app}$	Gene ID	% Change	mRNA FC	$\Delta\Delta G_{app}$
BCAM	-93.57%	0.37	7.47	APOB	-77.86%	1.22	-1.55
PTPRF	-90.54%	1.02	4.45	CNPY2	-71.51%	1.47	2.14
ITGA1	-88.31%	0.53	5.05	CDH2	-66.38%	N/A	-0.49
DNAJC3	-90.05%	2.68	0.01	C3	-72.51%	1.49	1.52
AGRN	-90.28%	N/A	5.25	SERPINA3	-67.36%	0.15	4.52
LAMB1	-90.17%	0.42	3.97	OAF	-59.33%	1.32	-0.19
PLOD2	-89.50%	0.32	1.76	ICAM1	-67.37%	0.35	2.49
MAN2B1	-81.21%	1.67	6.06	NEU1	-68.10%	1.91	4.43
FKBP9	-81.20%	0.97	4.07	COLGALT1	-66.09%	0.48	4.55
PLA2G15	-78.89%	0.63	4.02	GANAB	-65.09%	1.17	-0.02
PLOD3	-83.90%	0.99	5.40	PDIA5	-60.02%	2.10	1.58
PTK7	-83.94%	0.78	6.07	EMC1	-58.22%	0.86	0.15
SORT1	-82.00%	0.61	3.44	MLEC	-58.68%	2.33	2.78
LAMA5	-83.22%	0.64	1.48	IGF2R	-57.43%	1.64	11.07
LGMN	-79.19%	0.59	0.64	GGH	-50.55%	1.68	-0.28

Conclusions & outlook

Investigation of TL020's effect on host protein expression revealed that this analogue is likely less specific than CADA. A high percentage of the most down-regulated proteins contain N-terminal signal peptides (data not shown). To determine which proteins are being targeted in a signal peptide dependent fashion, follow up studies are planned. Each protein will be overexpressed with fluorescent tags in the presence of TL020 or DMSO control. Additional studies will be necessary to determine which proteins are responsible for affecting HBV replication. The work presented here will be useful in prioritizing proteins for these further investigations.

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1) Seto WK, Lo YR, Pawlotsky JM, Yuen MF. Chronic hepatitis B virus infection. *Lancet*, **2018**, 392(10161):2313-2324.
2) Vermeire K, Bell TW, Van Puyenbroeck V, Giraut A, Noppen S, Liekens S, Schols D, Hartmann E, Kalies KU, Marsh M. Signal peptide-binding drug as a selective inhibitor of co-translational protein translocation. *PLoS Biol.*, **2014**, 12(12):e1002011.
3) Hessa, T., Meindl-Beinker, N., Bernsel, A., Kim, J., Sato, Y., Lerch, M., Lundin, C., Nilsson, I., White, SH. and von Heijne, G. Molecular code for transmembrane-helix recognition by the Sec61 translocon. *Nature*, **2007**, 450, 1026-1030.