AN ANTI-LARGE T-ANTIGEN STRATEGY TO DEVELOP ANTI-JCV DRUGS

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ABSTRACT:

Introduction: There are currently no JCV-specific therapies available for clinical use. This study evaluates viral large T antigen (LTA) as a potential target for drug development. LTA is a hexameric protein with a helicase activity that is powered by ATP binding and hydrolysis. The helicase and ATPase function is critical for viral replication and inhibition by small molecules would disrupt the viral life cycle.

Methods: Recombinant JCV LTA was produced in *Escherichia coli* using a plasmid from Dr. K.Khalili. ATPase activity was measured using the malachite green assay. The K_m for ATP was determined (80nM) and the assay optimized for buffer components. A high throughput screen was completed using LDDN brain-biased compound library of 75,000 compounds. The compounds in the library were selected with filters for drug-like properties and physicochemical properties consistent with blood brain barrier permeability.

Results: Five compounds showed non-competitive inhibition of ATPase with an EC_{50}≤10μM. Modest antiviral activity was demonstrated in an immunofluorescence assay for JCV VP-1 expression in COS7 cells (EC50 15.1, 18.1, 20.0, 26.6, and 52.5 μm respectively). IC_{50} in the MTS96 and Cell TiterGlo assays was >100μM for all compounds in COS7 as well as HEK293 cells. However, two compounds inhibited cell proliferation in culture with IC50 values of 42.9 and 34.2 μM respectively. Three compounds inhibited viral replication in a real time PCR assay at concentrations between 10 and 100 μM, but cell replication was also proportionally affected.

Conclusion: LTA is a valid target for discovery of anti-JCV drugs. The hits identified can be starting points for medicinal chemistry to improve potency & selectivity. Screening of more libraries could also be considered to identify compounds that may be more potent with acceptable cytotoxicity.

INTRODUCTION: We hypothesize that JCV inhibitory and glial protective drugs can be discovered by screening chemical libraries for compounds that can inhibit the helicase machinery associated with JCV large T antigen (LTA). LTA is good target for drug discovery because (a) it is a key viral protein required for DNA replication, (b) it is well conserved across multiple viral strains, and (c) there is no homologous protein present in human cells, which offers of the prospect of developing anti-viral compounds with an acceptable toxicity profile. LTA directs the initiation of viral DNA replication by assembly into a double hexameric helicase which unwinds the duplex DNA bidirectionally. The initial step is a binding of LTA to the origin binding domain on the viral regulatory region. The progression of viral replication requires the recruitment of several cellular factors including human replication protein A (hRPA), DNA polymerase alpha-primase, and DNA polymerase delta. These biochemical changes are energy dependent, and an ATPase domain as well as an ATP binding site are present in the LTA protein. Hence, it is reasonable to expect that small molecule inhibitors of LTA ATPase and helicase activity will be detrimental to viral replication. Since, JCV replication caused oligodendroglial degeneration in PML, anti-JCV small molecules will have a protective effect on glial cells in this disease. The ATPase based assay described here is quite suited for high throughput screening, since it is a 'mix and measure' assay that requires only the addition of reagents. No extraction, centrifugation, or filtration steps are involved.

METHODS

Production of recombinant JCV LTA: To produce recombinant JCV LTA, the glutathione *S*-transferase (GST) fusion protein of the intronless JCV large T antigen (pGEX1-LTA)was been obtained from Dr Kamel Khalili at Temple University, Philadelphia. Ten-milliliter overnight cultures of *Escherichia coli* BL21 (*E. coli* B. F-, *omp*T, *hsd*S, *gal*, *dcm*) transformed with pGEX1λ-LTA were diluted 1:10 in fresh YT (2x) medium supplemented with ampicillin (100 mg/ml). Cultures were induced with 1mM isopropyl-b-thiogalactopyranoside (IPTG) at an optical density of 0.6 and incubated overnight at 37˚ C.

Cells were collected by centrifugation and resuspended in 10ml of CelLytic B per gram of cell paste, supplemented with 0.2 mg/ml of lysozyme, 50 units/ml of benzonase, 1 μM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (*Sigma-Aldrich*). After incubating the extraction suspension 20 min with shaking at room temperature, clear cell lysates were prepared by centrifugation at 12,000g for 10 min. Soluble and insoluble fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining.

High throughput screening for inhibitors of ATPase activity: Inhibitor of ATPase activity was assessed by the malachite green assay in which binding of molybdate and free phosphate produces a color change. The K_m for ATP was determined and the assay was optimized for buffer components including, NaCl, $MgCl_2$, $MnCl_2$, pH, detergent, and DTT. Then the linear range of the assay for different enzyme concentrations was determined in order to select an enzyme concentration within the linear range when the ATP concentration was at the K_m.

The assay was then miniaturized to 384-well plate format and automated for high throughput screening. The LDDN brain-biased small molecule library of 75,000 compounds was used for HTS. The compounds in the library were selected with filters to conform to Lipinski-type parameters but also to have the physicochemical properties to be more likely to cross the blood brain barrier.

Q-PCR Assay for Measuring JCV Replication: Real time PCR was used to directly measure JCV replication in Cos-7 cells. Simultaneous quantitation of a housekeeping gene ribosomal protein 32 (RPL32) sequence allowed monitoring of host cell replication. A 50% effective drug concentration (EC50), a 50% inhibitory (toxic) concentration (CC50), and the selectivity index (SI) were determined by standard methodology. The primers and probes used in this assay are directed to JCV VP-1 protein.

Fluorescent Focus Assays for Measuring JCV VP-1 Gene Expression: Seventy percent confluent Cos-7 cells in tissue culture plates with coverslips were infected with JCV Mad-4 stock for 4 days at 37C. Cells were fixed with 50% methanol-50% acetone for 10 min at room temperature, air dried for 15 min, and incubated for 1 hour at 37˚ C with anti-VP-1 antibodies in PBS, followed by fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin G secondary antibody. Quantitation of the percent of cells expressing viral antigens was performed using an in-house whole slide image analysis system. Drug concentrations effecting 50% reduction in VP-1 positive cells and total cell nuclei were respectively used to calculate EC50 and IC50.

RESULTS: The Km for ATP was determined and the assay was optimized for buffer components including magnesium, manganese, DTT, MOPS, and pH (Figures 1, 2)

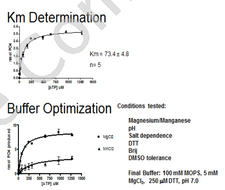

Figure 1: Km for ATP was 73,3 μM. The optimum buffer composition was 100mM MOPS, 5 mM MgCl2, 250uM DTT, pH 7.0.

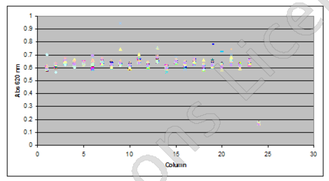

Figure 2: Reproducibility of the assay was satisfactory with a Z-factor of 0.76.

The most cost-effective LTA concentration and the optimal reaction time were determined (Figure 3). It was also ascertained that the ATPase assay was not sensitive to DTT at concentrations of up to 1200 μM and Brij concentrations of 0.1%. Five compounds LDN-0012723, 0015182, 0063710, 0060230 and 0065780 showed non-competitive inhibition of ATPase with an IC_{50}≤10μM (Table 1). Modest antiviral activity was demonstrated in an immunofluorescence assay for JCV VP-1 expression in COS7 cells (IC_{50} 18.1±8.2, 15.1±4.1, 52.5±17.7, 19.9±6.1, 26.5±16.3 μM respectively, expressed as mean ± SD)(see Table 2). However, LDN-0012723 and LDN-0015182 also inhibited cell proliferation with IC50 values of 42.9.1±2.6, and 34.2±8.2 respectively. Hence, the selectivity index of the anti-viral effect was low (SI= 2.4 and 2.3 respectively). The remaining three compounds did not inhibit cell proliferation at concentrations up to 50 μM. In toxicity assays not dependent on cell proliferation, namely the MTS96 aqueous and Cell TiterGlo assays, the IC50 was >100μM for all compounds in COS7 as well as HEK293 cells. Representative data is presented in for one compound in Figures 4 and 5. In quantitative real time PCR based assays designed to directly measure viral replication in COS7 and POJ cells, LDN-0063710 and LDN-0065780 showed no biologic activity, while the remaining three compounds inhibited viral and host cell replication proportionally at concentrations between 10 and 100 μM (data not shown).

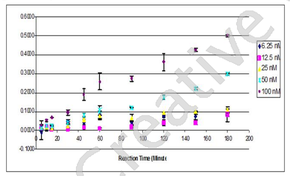

Figure 3: Time kinetics of inorganic phosphate release at different concentrations of LTA enzyme. The X-axis depicts time in minutes. The Y-axis plots the A620 nm of the reaction of phosphate with malachite green.

Table 1: ATPase Inhibitory Actibity of Selected Compounds

	EC50 (μM)	LD50 (μM) 6 day	
LDN#	ATPase	COS cells	HEK cells
LDN-0012723	0.8	93.8	71.8
LDN-0015182	4	>100	>100
LDN-0063710	5	>100	>100
LDN-0060230	10	>100	>100
LDN-0065780	15	>100	>100

Table 2: Antiviral Activity of Selected Compounds In Immunofluorescence Assay (mean +/- sd)

	IC50 μM	EC50 μM	SI
LDN-0012723	42.9 ± 2.6	18.1 ± 8.2	2.4
LDN-0015182	34.2 ± 8.2	15.1 ± 4.1	2.3
LDN-0060230	>50	20 ± 6.2	>2.5
LDN-0063710	>50	52.5 ± 17.7	>0.95
LDN-0065780	>50	26.6 ± 16.3	>1.9

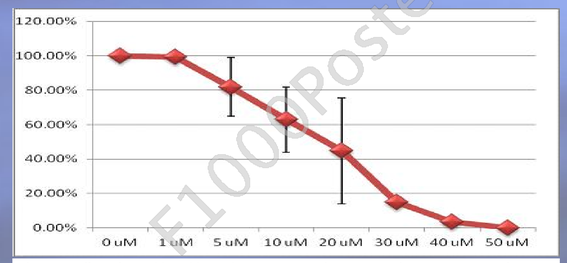

Figure 4: Effect of LDN 0015182 on JCV VP-1 expression expressed as a percentage of expression seen in cells not treated with drug. Error bars smaller than the width of the line graph are not visible.

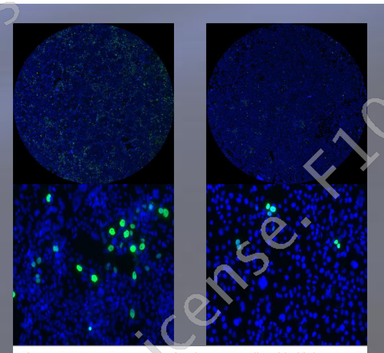

Figure 5. JCV VP-1 expression in Cos7 cells with (right panels) or without (left panels) exposure to 30 μM LDN 0015182. Image analysis was done to quantify the percentage inhibition of signal captured from the entire cover slip (upper panels). Higher magnification images (lower panels) illustrate that VP-1 staining had an exclusively nuclear localization.

CONCLUSIONS: LTA is a valid target for discovery of anti-JCV drugs. The hits identified are reasonable starting points for medicinal chemistry to improve potency and selectivity. Screening of additional chemical libraries could also be considered to identify chemical structures that may be more potent with acceptable cytotoxicity.

REFERENCE:

Gai, D.H., et al., Insights into the oligomeric states, conformational changes, and helicase activities of SV40 large tumor antigen. J Biol Chem, 2004. **279** (37): p. 38952-38959.