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Lysosomotropic Action of Amantadine: Basis for Treatment of COVID-19

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Abstract

SARS-coronavirus 2 is the causal agent of the COVID-19 outbreak. SARS-Cov-2 entry into a cell is dependent upon binding of the viral Spike (S) protein to cellular receptor and on cleavage of the spike protein by the host cell proteases such as Cathepsin L and Cathepsin B (CTSL/B). They are crucial elements of lysosomal pathway and both enzymes are almost exclusively located in the lysosomes. CTSL disruption offers potential for CoVID-19 therapies. The mechanisms of disruption include: decreasing expression of CTSL, direct inhibition of CTSL activity and modification of the CTSL environment (increase pH in the lysosome). We have conducted a high throughput drug screen gene expression analysis to identify compounds with the capacity to downregulate the expression of CTSL/CTSB. One of the most significant results shown to downregulate the expression of the CTSL gene is Amantadine(10uM). We confirmed Amantadine's lysosmal trapping capacity in an in-vitro Lysosomal Trapping Assay. In addition, to downregulating CTSL, Amantadine disrupts the lysosomal pathways, hence, interferes with the capacity of the virus to replicate. It acts as a lysosomotropic agent altering the CTSL functional environment. We propose that Amantadine could decrease the viral load in SARS-CoV-2 positive patients and as such it may serve as a potent therapeutic decreasing the replication and infectivity of the virus likely leading to better clinical outcomes. Clinical studies are currently needed to examine the therapeutic efficacy of Amantadine in COVID-19 infection.

Introduction

Recently a novel type of highly virulent beta-coronavirus was discovered in patients with pneumonia of unknown cause. Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV-2) as detected by sequencing of the samples was found to be the cause of a severe respiratory disease in humans [1]. The outbreak of COVID-19 resulted in a global epidemic with the number of confirmed cased surpassing 722, 000 in March 2020.

The SARS-CoV-2 genome shares about 80% similarities with SARS-CoV and is even more similar (96%) to the bat coronavirus BatCoVRaTG13 [2]. Corona viruses are characterized by large genetic diversity and frequent recombination of their genomes and hence pose a challenge in terms of public health, currently based on 1455 viral genomes and predicted 24.5 genetic substitutions per year [3].

Similar to SARS-CoV, SARS-Cov-2 enters the cell by the means of binding to cellular receptor(s) including the Angiotensin-Converting Enzyme 2 (ACE2) membrane bound protein [4]. Host protease dependence of SARS-CoV-2 entry is a critical step. SARS-CoV takes advantage of the endosomal cysteine proteases CTSL and CTSB [5,6]. CTSL is a peptidase that preferentially cleaves peptide bonds with aromatic residues in P2 and hydrophobic residues in the P3 position [7]. CTSL is active at pH 3-6.5, in the presence of thiol and its enzymatic stability is dependent on ionic strength [7]. CTSL proteolysis is a crucial mechanism for Ebola as well as SARS-CoV for processing of viral glycoprotein before cell membrane fusion [6]. Specifically, during cell membrane fusion, the S protein is cleaved by host cell proteases, exposing a fusion peptide of the S2 domain. This leads to the fusion of viral and cellular membranes and the release of the viral genome into the cytoplasm of the host cell.

Cleavage at both sites is believed to be necessary for viral entry by endocytosis into the host cell. The S1/S2 cleavage site of SARS-CoV-2 is between the threonine and methionine at positions 696 and 697. This S1/S2 cleavage site is identical to that of SARS-CoV which has been shown to be cleaved by CTSL, a lysosomal cysteine protease encoded by the CTSL1 gene. SARS-CoV-2 also has a furin-like protease cleavage site not found in SARS-CoV, between the arginine and serine at positions 685 and 686. This site may be cleaved by furin during viral egress. Interfering with the spike protein processing by the host cell, whether by affecting the environment or modulating gene expression levels, hence offers a potential therapeutic strategy.

Genetic variants within CTSL gene could in theory affect the propagation capacity of the virus. Furthermore CTSL polymorphisms could affect the susceptibility to SARS-CoV-2 where for example individuals with certain genetic variant have reduced expression of CTSL and in turn could be protected or have lower viral titers. Additionally, elements of hosts Major Histocompatibility Complex I (MHC I) and cytotoxic T Cell Lymphocytes (CTL)-mediated immune responses might affect viral proliferation [8]. There are susceptibility factors ranging from ethnicity background to age related groups, to comorbid conditions [9,10].

In a report of results of an earlier segment of our investigations we tested compounds that could help identify potential therapeutic agents with the capacity to decrease expression or inhibit the expression of the CTSL gene [11]. We identified Amantadine among top of the list of significant compounds. We now further confirmed Amantadine's lysosmal trapping capacity in an *in-vitro* Lysosomal Trapping Assay. Together these results provide a large body of evidence suggesting potential efficacy of Amantadine in treatment of COVID-19.

Materials and Methods

Cell culture and drug treatment

Drugs screening was carried out, the same one as applied in our

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Table 1: List of top drugs affecting transcriptional CTSL downregulation (log2 of normalized Affymetrix probe intensity.

Drug ID	log2(treated) CTSL	log2(treated) CTSL	log2 difference
Baclofen	9.58	10.4	-0.82
Triprolidine Hydrochloride	9.54	10.33	-0.79
Brompheniramine Maleate	9.57	10.33	-0.75
Amantadine Hydrochloride	9.62	10.33	-0.7
Phenytoin	9.56	10.26	-0.7
Atropine Sulfate	9.63	10.33	-0.7

Table 2: Top lysosomal ontology terms over-represented among transcriptionally downregulated genes treated with Amantadine.

Term	Count (n)	% Pathway	Fold Enrichment	*P*-value	*P*-value Bonferroni	*P*-value FDR
GO:0005764~lysosome	21	5.19	4.42	6.65E-08	2.49E-05	9.19E-05
Lysosome	19	4.69	3.75	3.77E-06	0.001389	0.005193
hsa04142:Lysosome	14	3.46	4.37	1.55E-05	0.003425	0.019698

previous study [12]. The retinal pigment epithelia cell line, ARPE-19/HPV-16, was chosen to establish a database of drug profiles because of its non-cancerous, human origin, with a normal karyotype. It can also be easily grown as monolayer in 96-well plates. Compounds were obtained from Sigma (St. Louis, MO) or Vanda Pharmaceuticals (Washington, DC). Cells were aliquoted on 96-well plates (~2×10e5 cells/well) and incubated for 24 h prior to providing fresh media with drug, or the drug vehicle (water, dimethyl sulfoxide, ethanol, methanol, or phosphate-buffered saline solution). Drugs were diluted 1000 fold in buffered in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (D-MEM/F-12) culture medium (Invitrogen, Carlsbad, CA) containing nonessential amino acids and 110 mg/L sodium pyruvate. In these conditions, no significant changes of pH were expected, which was confirmed by the monitoring of the pH indicator present in the medium. A final 10 μM drug concentration was chosen because it is believed to fit in the range of physiological conditions [12]. Microscopic inspection of each well was conducted at the end of the treatment to discard any samples where cells had morphological changes consistent with apoptosis. We also verified that the drug had not precipitated in the culture medium.

Gene expression

Cells were harvested 24 h after treatment and RNA was extracted using the RNeasy 96 protocol (Qiagen, Valencia, CA). Gene expression for 22,238 probe sets of 12,490 genes was generated with U133A2.0 microarrays following the manufacturer's instructions (Affymetrix, Santa Clara, CA). Drugs were profiled in duplicate or triplicate, with multiple vehicle controls on each plate. A total of 708 microarrays were analyzed including 74 for the 18 antipsychotics, 499 for the other 448 compounds, and 135 for vehicle controls. The raw scan data were first converted to average difference values using MAS 5.0 (Affymetrix). The average difference values of both treatment and control data were set to a minimum of 50 or lower. For each treatment category, all probe sets were then ranked based on their amplitude or level of expression relative to the vehicle control (or the average of controls was selected when more than one was used). Amplitude was defined as the ratio of expression (t–v) / [(t+v) / 2] where t corresponds to treatment instance and v to vehicle instance.

In vitro hepatocyte lysosomal trapping studies

This protocol was designed to evaluate Amantadine for lysosomal trapping potential in immortalized hepatocytes (Fa2N-4 cells). Fa2N-4 cells are immortalized human hepatocytes that retain expression and function of lysosomes and can be used to evaluate accumulation of compounds in lysosomes. Specifically, the test article was incubated with Fa2N-4 cells in the presence or absence of ammonium chloride (an inhibitor of lysosomal trapping). The amount of test article that accumulates in the cells was quantified by LC-MS/MS. Incubations of propranolol (a known lysosomotropic drug) with and without ammonium chloride were used as positive controls.

Results

Drug screening

With the aim of discovering potential pharmaceutical agents capable of affecting transcriptional expression levels of CTSL implicated in SARS-CoV and SARS-CoV2 Pathophysiology, we screened 466 compounds belonging to 14 different therapeutic classes. Screening was conducted using human retinal pigment epithelia cell line (ARPE-19) and gene expression changes were collected across 12,490 genes. The ARPE-19 cell line was initially selected as a well suited model for the study of compounds that affect neuronal type cells, in particular antipsychotics. Here, we describe the discovery of a CTSL/B, lysosomotropic signature which might give insights into the therapeutic potential of the tested compounds.

We analyzed the expression profiles of CTSL across all 466 compounds tested. In order to find positive hits we selected only those results that showed a reduction of expression of CTSL (1.5 -fold difference).There were no drugs that decreased CTSL expression by more than 40%. Between the most 5 potent compounds were drugs from various therapeutic areas - muscle relaxant, antihistamine, anti-epileptic, anticholinergic and antiviral (Table1). Top results (top 5 of 466) included Amantadine hydrochloride, an established and safe antiviral agent that was previously used to treat patients with influenza A.

Due to its high lipophilicity, Amantadine can cross lysosomal membranes and accumulate in lysosomes acting at higher micromolar concentrations as lysosomotropic alkalinizing agent [9-13]. Amantadine inhibits influenza A replication at low micromolar concentration, by blocking M2 ion channel protein which acidifies the virus interior and releases its nucleoprotein [9-14]. It causes pH alteration which ultimately abrogates membrane fusion a necessary step for virus replication [13,14]. Other lysosomotropic drugs affect lysosomes through lysosome membrane permeabilization and

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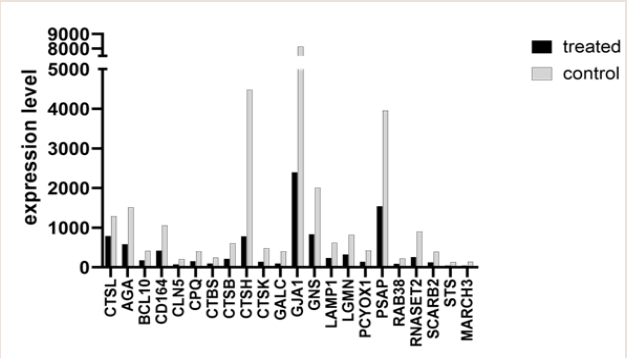

Figure 1: Notable difference in over-representation of genes with downregulated expression across lysosome pathway genes. Black columns represent average expression levels of Amantadine treated samples, gray columns represent average expression levels of control samples. Y-axis represents Affymetrix probe intensity associated with expression.

accumulation also blocking of Ca^{2+} signaling, and enzyme activity inhibition orstorage material accumulation [15]. Since Amantadine behaves as a lysosomotropic substance that passes easily through the lysosome membrane of SARS-CoV-2 virus and accumulates, where it could increase the pH of lysosome and thus inhibit the protease activities [15]. Moreover Amantadine may directly affect viral entry by down-modulating CTSL and other lysosomal pathway genes. The PK profile of the drug makes it particularly suitable for administration to humans. Plasma concentration is in the range of 200-800 ng/mL depending on the formulation and dosing regimen. Plasma half-life is 17 h (range: 10-25 h) with renal clearance as main elimination mechanism. Amantadine HCl [immediate release] is available as a 100-mg tablet and 50 mg/5 mL syrup and is typically administered twice daily [16]. Human cells in tissue culture readily tolerated Amantadine up to a concentration of 100 ug/mL (~657 uM).

Since CTSL was not the top differentially-expressed transcript, we decided to extend our analysis to all the genes that were downregulated by Amantadine. Among the top 500 differentially expressed probes (383 genes, all with at least 50% expression reduction) we have found 21 genes related to lysosomal terms using David Enrichment tool (GO:005764, p=2.49x10-5). Moreover, the top significant pathway by ENRICHR enrichment analysis toolwasthe KEGG lysosome pathway. Amantadine's significant effect upon lysosome pathway genes is shown on Figure 1 and Table 2. Figure 1 displays notable difference in over-representation of genes with downregulated expression across lysosome pathway genes. Table 2 displays top lysosomal ontology terms over-represented among transcriptionally downregulated genes treated with Amantadine.

Lysosomal trapping assay

Lipophilic and amphiphilic drugs with ionizable amines can accumulate in lysosomes - a process known as lysosomal trapping [17]. To test the capacity of Amantadine to act as lysosomotropic agent we have conducted an *in-vitro* hepatocytes lysosomal trapping assay as described previously in literature [17,18]. We focused on the difference in uptake and hence the amount of test compound trapped in lysosomes. We confirmed that Amantadine showed lysosomal uptake and lysosomal trapping capacity. Circa 50% of Amantadine was trapped in lysosomes at 1 μM and showed significant saturation at higher concentrations. Specifically, the uptake of Amantadine (1, 10 and 100 μM) in Fa2N-4 cells was concentration dependent and was reduced up to 46.7 and 40.5% at 1 μM in the presence of ammonium chloride at the 10 and 30 min incubation period, respectively (Supplemental Figure 1). The uptake was marginally reduced at 10 and 100 μM. These results suggest the potential for lysosomal trapping at low concentration. Hence, the experiments confirmed Amantadine's capacity to act as a lysosomotropic agent.

Discussion

Decreasing the expression of CTSL is likely a potential mechanism that would lower the capacity of the virus to enter the next host cell. Another symbiotic, therapeutic mechanism is lysosomal pH modulation that would further interfere with proteolytic spike protein activation. Therapeutic agents capable of perturbing the lysosomes, their function or microenvironment may offer protection from the virus or decrease the severity of the symptoms. Given that Amantadine not only down-regulates CTSL expression, but a number of key lysosomal enzymes, we can now hypothesize that lysosomal dysfunction induced by Amantadine administration could be protective against viral entry and ultimately replication. Our hypothesis is that people with certain lysosomal storage diseases may be resistant to one of these viruses. Along these lines there is suggestive evidence for this to be true. For example Niemann-Pick disease type C1 lipid storage disorder offers resistance to Ebola in patient cell lines [19,20]. Interestingly, bat species show selective sensitivity to Ebola versus Marburg viruses [21].

Interfering with the lysosomal milieu can have protective effects from coronavirus which we know uses CTSL, a pH sensitive enzyme, to process the cleavage of the spike protein. Amantadine would be predicted by physical and chemical properties to accumulate in the lysosomes and raise pH, interfering with CTSL function. The gene expression pattern reported in this paper suggests that a more general lysosomal program is down-regulated by Amantadine, likely through a common set of transcription factors. Additionally, Amantadine's property to accumulate in lysosomes, if effective, could reduce viral load, decrease intra-host organ spread and decrease patient-associated disease severity and progression. Importantly, the dose of Amantadine that was tested in High Throughput Screen Assay is within one order of magnitude of expected pharmacokinetic, clinical profile (~5 uM). That would mean the drug can be administered per existing, safe and approved label dosing.

Further studies including clinical trials are now required in order to examine the role of Amantadine administration as a treatment for COVID-19.

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