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SARS-CoV-2 E protein is a potential ion channel that can be inhibited by Gliclazide and Memantine

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ABSTRACT

COVID-19 is one of the most impactful pandemics in recorded history. As such, the identification of inhibitory drugs against its etiological agent, SARS-CoV-2, is of utmost importance, and in particular, repurposing may provide the fastest route to curb the disease. As the first step in this route, we sought to identify an attractive and viable target in the virus for pharmaceutical inhibition. Using three bacteria-based assays that were tested on known viroporins, we demonstrate that one of its essential components, the E protein, is a potential ion channel and, therefore, is an excellent drug target. Channel activity was demonstrated for E proteins in other coronaviruses, providing further emphasis on the importance of this functionally to the virus' pathogenicity. The results of a screening effort involving a repurposing drug library of ion channel blockers yielded two compounds that inhibit the E protein: Gliclazide and Memantine. In conclusion, as a route to curb viral virulence and abate COVID-19, we point to the E protein of SARS-CoV-2 as an attractive drug target and identify off-label compounds that inhibit it.

1. Introduction

Coronaviruses are positive-sense, single-stranded RNA viruses that are often associated with mild respiratory tract infections in humans [1]. However, three members of the family have received notoriety due to their abnormal virulence: SARS-CoV was the etiological agent of the SARS epidemic in the winter of 2002/3 that caused 774 deaths amongst 8098 cases; MERS-CoV was responsible for the MERS epidemic that started from 2012 with 862 deaths from 2506 infections; Finally, SARS-CoV-2 is responsible for the ongoing COVID-2019 pandemic resulting in 464,510 deaths out of 8,794,337 cases.[1]

Genomic analyses have indicated that SARS-CoV and SARS-CoV-2 are very similar to one another (ca. 80%) but are distinct from most other Coronaviridae members that infect humans. Both viruses have been placed in subgroup B in the Betacoronavirus genus within the Orthocoronavirinae subfamily of the Coronaviridae [2–4].

Repurposing known drugs against essential components of SARS-CoV-2 may represent the most rapid solution to curb COVID-19. Therefore we sought to identify attractive drug targets in the virus. Since ion channels are excellent drug targets, our focus shifted to a potential viroporin in the virus - its E protein.

Of all coronavirus structural proteins, E is the least understood in terms of mechanism of action and structure. Functionally, the E protein has been implicated in viral assembly, release, and pathogenesis (the reader is referred to a recent excellent review [5]). Yet crucially, coronavirus E proteins are important for viral pathogenesis [6], and attenuated viruses lacking the protein have even been suggested to serve as vaccine candidates [7–10].

Their small size and overall hydrophobicity prompted suggestions that coronavirus E proteins might be viroporins. Indeed, E proteins from several coronaviruses: including SARS-CoV-1 [11–17], MERS coronavirus [18], human coronavirus 229E [15], mouse hepatitis virus [15], and infectious bronchitis virus [15,19], were shown to posses cation selective channel activity that may be blocked by Hexamethylene amiloride [13,15].

The importance of ion channel functionality for virulence has been demonstrated in several viruses. For example, the H^+ channel activity of the M2 protein of influenza A is critical to infectivity in two ways: Following endocytosis, the channel facilitates H^+ [20] and K^+ transport [21] into the viral lumen, enabling viral RNA to dissociate and initiate replication. In addition, M2 may also prevent

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[1] As of June 21, 2020 according to the Coronavirus COVID-19 Global Cases by Johns Hopkins CSSE.

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the acidification during Golgi transport, thereby abrogating the premature, irreversible conformational change of the HA fusogenic spike protein [22,23], if the activating proteolytic cleavage occurs.

In coronaviruses, the channel activity of the E protein was shown to be critical for infectivity. For example, pathogenicity was significantly hampered when the transmembrane segment of infectious bronchitis virus E protein was replaced with a heterologous domain that lacked ion channel activity [24]. In SARS-CoV-1 virus, studies have shown that viruses in which the channel activity of the E protein was abolished, were far less infectious [25].

The specific role of the channel functionally of the E protein in coronaviruses has been implicated in several stages. Once inside the host cells, new virions are formed by budding into the Golgi complex lumen or into the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) lumen. Subsequently, viruses are assembled into exocytotic vesicles for export from the host cell [26]. This cellular location is exactly where, significant amounts of the E protein are shown to exist during viral infection [24,27–31]. Interestingly, Enjuanes and colleagues have shown that SARS-CoV-1 E protein exhibits Ca^{2+} transport activity in the ERGIC, pointing to a direct role for the protein's channel [32]. Moreover, the authors have shown that this Ca^{2+} conductivity activates the NLRP3 inflammasome, a finding that correlates well with the medical symptoms of the disease.

Taken together, three premises motivated us to examine if SARS-CoV-2 E protein is an ion channel: (i) E proteins from several coronaviruses were shown to posses channel functionality [13,15,16,18]; (ii) Ion channels are excellent targets for pharmaceutical point inhibition; and (iii) Coronavirus E proteins are important for viral virulence. Hence any inhibitor/channel-blocker found against the SARS-CoV-2 E protein may serve as an anti-COVID-19 drug candidate.

2. Results

Three, recently developed bacteria-based assays [33–35] were used to examine if SARS-CoV-2 E protein is an ion channel. These assays are quantitative, easy to implement rapidly, and are amenable to high-throughput screening for inhibitor identification. Moreover, each of these therethree assays was used on known viroporins and were shown to distinguish non-conducting transmembrane domains [36]. Finally, one of the assays can also be used to predict, prior to clinical use, the options that the virus has to develop resistance against any particular inhibitor of the channel [37].

In order to ensure proper membrane incorporation, we made use of the pMAL protein fusion and purification system (New England Biolabs). In this construct which has been used successfully with multiple viroporins [33–36], SARS-CoV-2 E protein is fused to the carboxyl terminus of the periplasmic maltose binding protein. As a positive control, we compared the activity of the E protein to the M2 channel from the influenza A virus, the archetypical viroporin that can be inhibited by aminoadamantanes [20].

2.1. Negative genetic test

The first test that was undertaken was to examine if the SARS-CoV-2 E protein's channel activity can lead to membrane permeabilisation and thereby negatively impact bacterial growth (negative genetic test). As seen in numerous other viroporins [33–36], when expressed at increasing levels, channel activity hampers growth due to its deleterious impact on the proton motive force. Subsequently, channel-blocking drugs may be identified readily due to their ability to alleviate growth retardation. The data in Fig. 1 show that expression of the SARS-CoV-2 E protein causes significant bacterial growth retardation proportional to the protein's expression levels. This behaviour is similar to that of a known proton channel, the M2 influenza A protein.

We recognize that growth retardation is not an uncommon consequence of heterologous protein expression in bacteria. In other words, spurious factors could lead to bacterial death in addition to channel activity. We, therefore, demonstrate that the bacterial death obtained in the negative assay is due to the protein's channel activity in the following three ways. (i) We identify E protein channel blockers and show that they can revive bacterial growth. (ii) We developed a complementary bacterial growth assay, where channel activity is essential for growth (positive genetic test). (iii) We show that protein expression increases H^+ conductivity in an assay involving a pH-sensitive GFP [34].

2.2. Positive genetic test

The second experimental test that we have performed examines K^+ conductivity. Specifically, K^+-uptake deficient bacteria [38] are incapable of growth, unless the media is supplemented by K^+. However, when a channel capable of K^+ transport is heterologously expressed, the bacteria can thrive even under low K^+ media [35,36]. Hence, in this instance the viral channel is essential to bacterial growth (positive genetic test). Finally, results shown in Fig. 2 indicate that expression of SARS-CoV-2 E protein is able to increase the growth rate of K^+-uptake deficient bacteria in otherwise limiting conditions (i.e., low [K^+]).

2.3. Fluorescence-based test

The final test to examine channel activity, was based on detecting protein-mediated H^+ flux in bacteria that express a pH-sensitive green fluorescent protein [34]. The addition of an acidic solution to the media will result in cytoplasmic acidification if the bacteria express a channel capable of H^+ transport. Consequently, as seen in Fig. 3, expression of the E protein from SARS-CoV-2 results in appreciable cytoplasmic acidification, indicative of its ability to transport protons. Similar acidification was detected in other viroporins, such as the influenza A M2 channel [34,36].

2.4. Screening for channel blockers

Considering that all three bacterial assays indicated that the SARS-CoV-2 E protein is a potential viroporin, we set forth to screen a small data set of known channel blockers. We selected a library of 372 compounds from MedChemExpress (NJ, USA) in the area of "Membrane Transporter/Ion Channel". Each of these chemicals was tested in the positive and negative genetic tests detailed above.

In the negative assay, bacteria experience appreciable growth retardation due to the expression of the SARS-CoV-2 E protein at elevated levels (Fig. 1). Therefore, channel blockers can be readily identified since they alleviate this growth retardation. Note that this screen inherently reduces potential toxicity since it selects for chemicals that are not toxic to the bacteria. Specifically, each of the chemicals in the pilot library was added to the media, followed by growth recording and comparison to bacteria that did not receive any treatment. Out of the 372 compound drug library, several chemicals relieved the growth inhibition that the bacteria experienced due to the SARS-CoV-2 E protein activity. Of particular notice are Gliclazide and Memantine that enhance bacterial growth, as shown in Fig. 4a.

In the positive assay screening, a reciprocal picture is obtained. K^+-uptake deficient bacteria grown in low [K^+] media experience growth enhancement due to the (low level) expression of the SARS-CoV-2 E protein (Fig. 2). Therefore, channel blockers can be

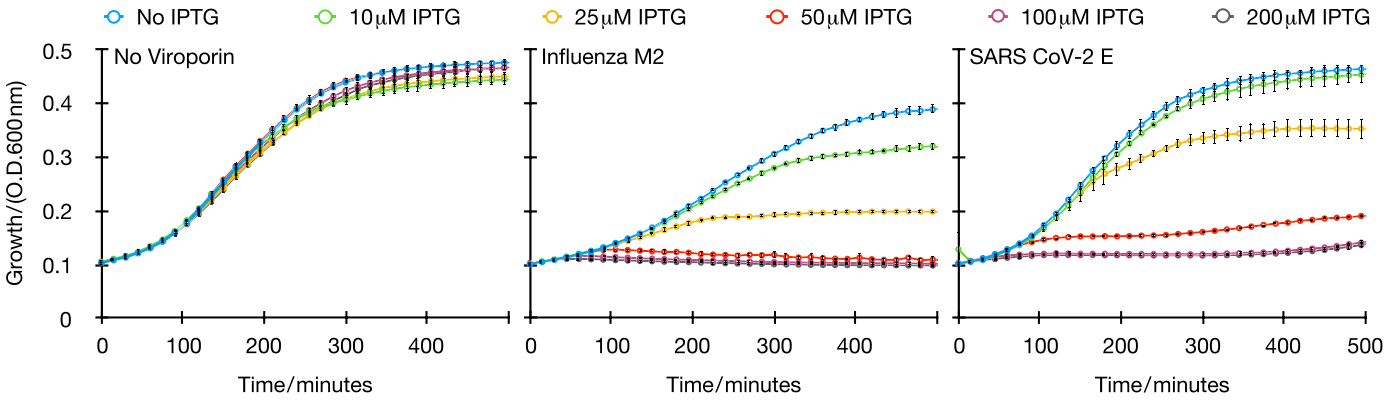

Fig. 1. Positive genetic assay. Growth curves (n = 2) of bacteria as a function of SARS-CoV-E protein expression (right). Bacteria that express the maltose binding protein with out a conjugated viral ion channel are shown in the left panel as a negative control. Bacteria that express the influenza M2 viroporin, as a positive control, are shown at the center. Induction at different IPTG concentrations (as noted), takes place when the bacteria density reaches an O.D.600 nm of 0.1. Growth O.D.600 nm values were collected every 15 min.

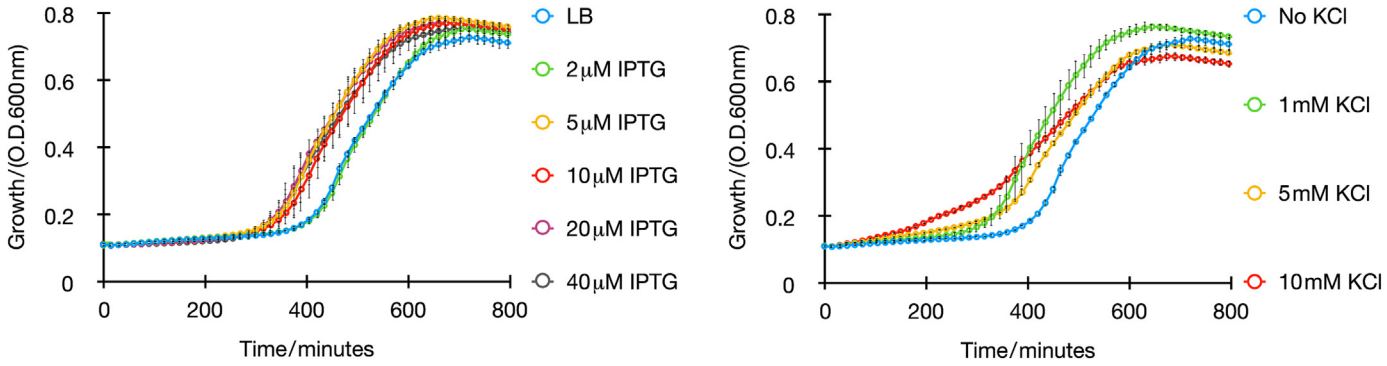

Fig. 2. K^+ conductivity assay. Impact of viral protein on the growth of K^+-uptake deficient bacteria [38] (left panel). Different protein expression levels are achieved by varying the concentration of the IPTG inducer, as noted. Bacterial growth rate as a function of $[K^+]$ is plotted in the right panel.

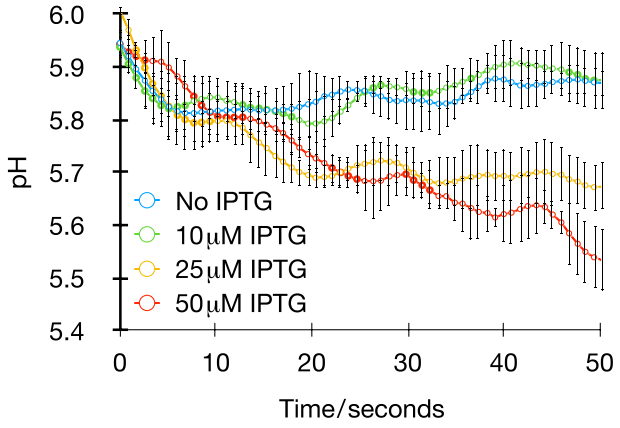

Fig. 3. Fluorescence-based H^+conductivity assay. The fluorescence of bacteria that harbour pHluorin, a pH sensitive GFP [39], was examined as a function of SARS CoV-2 E protein expression [34,40]. Protein levels were governed by the level of the inducer (IPTG) as indicated. The results are an average of two independent experiments, with standard deviations depicted as error bars.

identified since they result in growth retardation. In a manner similar to the negative assay, each of the chemicals in the pilot library was added to the media followed by growth recording. Once again, Gliclazide and Memantine scored positively in the test, in that they both inhibited growth, (Fig. 4b).

3. Discussion

Three independent and complementary assays have indicated that SARS-CoV-2E protein is a potential ion channel. Each one of the bacteria-based assays was tested extensively on known viroporins as positive controls [33–35], as well as exhibiting negative results when analyzing non-conductive proteins [36]. Over expression resulted in bacterial death due to membrane permeabilisation (Fig. 1). Conversely, the protein was able to rescue growth of K^+-uptake deficient bacteria (Fig. 2). Finally, SARS-CoV-2 E protein expression was able to change the fluorescence of bacteria that express a pH-sensitive GFP (Fig. 3). The latter two assays indicate that the protein conducts K^+ and H^+s. Cation conductivity has also been shown for other coronaviruses E proteins [11–19], providing further credence to the assigned channel functionally of the E protein form SARS-CoV-2.

The importance of the channel functionally of the E protein in SARS-CoV-2 may be ascribed from other coronaviruses, and in particular the highly similar SARS-CoV-1. As discussed in the introductory section, the channel activity of the protein is essential for virulence [24,25] and in particular, Ca^{2+} conductivity in the endoplasmic reticulum-Golgi intermediate compartment [32].

Having established that the E protein is a potential channel, and as such a viable drug target, we examined the ability of a small screen to identify inhibitors thereof (Fig. 4). Two chemicals scored positively in reciprocal assays ruling out any spurious factors. When the E protein is detrimental to bacteria, the chemicals enhanced growth. However, when the E protein is essential to bacteria, the

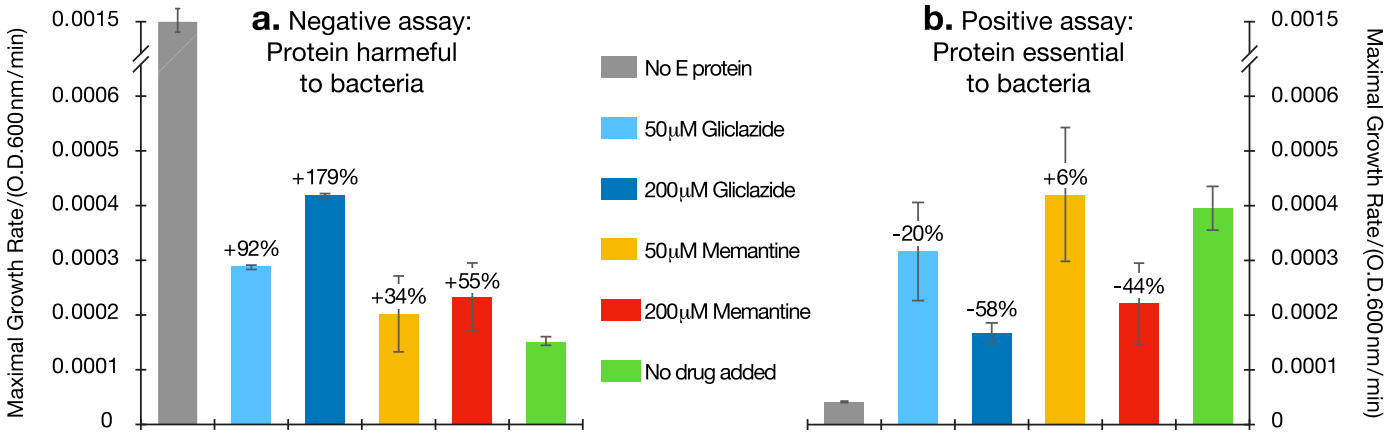

Fig. 4. Compound screening results using the positive and negative genetic tests. Impact of different drugs, as noted, and E protein expression on the growth rates of bacteria. a. Negative genetic test in which SARS-CoV-2 E protein is expressed at an elevated level (40 μM [IPTG]) and is therefore deleterious to bacteria. In this instance inhibitory drugs enhance bacterial growth. b. Positive genetic test in which SARS-CoV-2 E protein is expressed at low level (10 μM [IPTG]) in K^+-uptake deficient bacteria. In this instance inhibitory drugs reduce bacterial growth. In both panels the impact on growth in comparison to growth without any drug is listed.

same compounds were deleterious to growth.

In conclusion, our results demonstrate that the SARS-CoV-2 E protein is a potential ion channel. Since coronavirus E proteins are essential to virulence [6–10], it represents an attractive drug target. Moreover, our screening efforts identified two inhibitors that block E protein channel activity, thereby strengthening the validation of such a route to curb COVID-19.

4. Materials and methods

4.1. Channel assays

All three bacteria-based assays were conducted as described previously [36]. The only minor differences were as follows: Protein induction ranges differed in the assays, as noted in Fig. 1–3. Furthermore, in the fluorescence-based assay protein induction took place for 2 h prior to measurement.

4.2. Chemical screening

A library of 372 transporter/ion channel blockers was purchased from MedChem Express (HY-L011, Monmouth Junction, NJ). Each chemical was added at various concentrations to the growth media with a total concentration of Dimethyl sulfoxide not exceeding 1%. All manipulations and growths were conducted on a Tecan EVO 75 robotic station (Männedorf, CH).

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Declaration of competing interest

The authors declare that they are in the process of filing a patent for second medicinal use of Gliclazide and Memantine as anti COVID-19 agents.

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