

# Amantadin has potential for the treatment of COVID-19 because it targets known and novel ion channels encoded by SARS-CoV-2

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## Research Article

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# Abstract

The dire need for therapies against SARS-CoV-2 infections is obvious and inspires strategies of repurposing drugs approved for other indications. Current examples are remdesivir (originally developed for ebola treatment) and steroids (anti-inflammatory treatment). Here we propose to use amantadine (an anti-influenza A drug) as a novel, cheap, readily available and effective way to treat COVID-19 because of its ability to inhibit known (Protein E) and novel (Orf10) ion channels identified in the virus genome.

## Main Text

Ion channels are excellent drug targets as exemplified by multiple drugs controlling the cardiovascular system, locomotion and various functions in the central nervous system<sup>1,2</sup>. In their genomes, viruses may encode ion channels, denoted viroporins, formed by oligomerization of transmembrane units<sup>3</sup>. Over the past decades, an increasing number of both cationic and anionic viroporins have been identified and are thought to play central roles in virus life cycle, in addition to having a huge impact on the virus-mediated pathologies in the host<sup>3</sup>. Viroporins have been identified in a vast amount of pathogenic viruses including hepatitis C (HCV), HIV-1 and influenza A viruses as well as picornaviruses and coronaviruses<sup>3</sup>. Several drugs have been presented including marketed drugs, such as amantadine and rimantadine, which target the M2 ion channel in influenza A virus<sup>4</sup>.

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the cause of the ongoing pandemic of COVID-19. It is highly homologous to the deadly SARS-CoV-1, giving rise to "SARS" in 2002 and MERS-CoV, giving rise to Middle East Respiratory Syndrome in 2012<sup>5</sup>. One conserved viroporin has been identified in these three coronaviruses, the homo-pentameric cationic Protein E, while the homo-tetrameric cationic Protein 3a is found in SARS-CoV-1 and -2 (Fig. 1). In SARS-CoV-1, expression of both viroporins promotes virus replication and virulence<sup>6</sup> and deletion of Protein E gene attenuates the virus, resulting in faster recovery and improved survival in infected mice<sup>7</sup>. At the cellular level, deletion of Protein E decreases edema accumulation, the major determinant of the deadly acute respiratory distress syndrome (ARDS) in addition to reducing levels of inflammasome-activated IL-1b, indicating that E protein ion channel function is required for inflammasome activation<sup>7</sup>. Hence, Protein E ion channel activity represents a determinant for SARS-CoV-1 virulence, and mirrors the pathology associated with the severe cases of SARS-CoV-2 infection. This suggests that inhibition of the SARS-CoV-2 E viroporin will limit pathogenicity and be of therapeutic value to SARS-CoV-2 infections.

Recently, the structure of the transmembrane domain of Protein E (ETM) from SARS-CoV-2 was solved<sup>8</sup>. In lipid bilayers, ETM forms a five-helix bundle surrounding a narrow pore similar to the homologous Protein E from SARS-CoV-1<sup>9</sup>. Interestingly, the anti-influenza drug, amantadine has previously been shown to bind to the native transmembrane  $\alpha$ -helical domain of SARS-CoV-1 Protein E with an affinity comparable to that observed for the influenza A virus M2-channel<sup>4</sup> and was also shown to directly inhibit SARS-CoV-1 Protein E mediated conductance<sup>10</sup>. Intriguingly, ETM from SARS-CoV-2 is 100% identical to

that of SARS-CoV-1, suggesting that it possesses a preserved amantadine binding site, as also suggested by modeling of the ETM of SARS-CoV-2<sup>8</sup>.

We looked into the genome of SARS-CoV-2 and identified two potential viroporins in open reading frame 7b (Orf7b) and Orf10 (Fig.1). Orf7b has been found to localize to the Golgi compartment as a membrane integral protein and be incorporated into SARS-CoV-2 particles<sup>11</sup>. We discovered amino acid sequence homology of the core of Orf7b to Protein E in a consensus region containing three conserved Phe-residues, all facing the lipid membrane, among which Phe23 and Phe26 are involved in helix-helix interactions in the homo-pentameric bundle<sup>8</sup>. Moreover, the Orf7b amino acid sequence motif (IIFWFSL) at the C-terminus of its predicted TM-region is highly homologous to a sequence (IIFWFLL) at the N-terminus of the TM-region of Protein E of transmissible gastroenteritis virus (TGEV). Orf10 is a 38 amino acid long peptide, the gene of which is positioned at the 3'-end of the viral genome. It is also present in Pangolin-CoV (with approximately 97% sequence homology) and in a truncated version in bat-, civit and SARS-CoV-1 genomes<sup>12</sup>. The truncated versions have only the first 29 amino acids, which according to our model (Fig.1) still leave the TM-region and thereby the protein functionally intact. In a limited genome study (339 sequences) in India it was observed, that while numerous mutations were observed in the rest of the SARS-CoV-2 genome, Orf10 (and Orf6) contained none and Protein E only one mutation, suggesting that these proteins could be potential targets for controlling the COVID-19 pandemic<sup>13</sup>.

To establish ion channel function of the two novel putative viroporins, Orf7b and Orf10, we employed the *Xenopus laevis* oocyte expression system and monitored current activity with conventional two-electrode voltage clamp. Oocytes expressing Orf7b and Orf10 presented with a significantly augmented current activity as compared to control oocytes (Fig.1). We also confirmed the ion channel activity previously described for Protein E and Protein 3a (Fig.1). Besides amantadine (Fig.2a-c), several other marketed and experimental compounds have been described as inhibitors of viroporins<sup>4</sup>. We selected seven compounds and monitored the ion channel activity of the four viroporins in the presence of 10  $\mu$ M of each drug. Four of these, rimantadine, adamantane, pyridin B and -Y had no effect, while three (amantadine, emodine and xanthene) attenuated at least two of the ion channels (Fig. 2d). Among these, the strongest effect was observed for amantadine in its inhibition of Protein E and Orf10 ion channel activity. In the presence of amantadine, the current in the Protein E (Fig. 2b) and Orf10 (Fig. 2c) expressing oocytes was reduced (Fig. 2bi, compare  $531 \pm 112$  nA for control solution with  $123 \pm 33$  for amantadine treatment at  $V_m = -85$  mV,  $n = 9-10$ ,  $p < 0.01$  and Fig. 2ci,  $401 \pm 48$  nA for control solution and  $137 \pm 18$  nA for amantadine treatment at  $V_m = -85$  mV,  $n = 9-10$ ,  $p < 0.001$ ). These results show that amantadine suppresses Protein E and Orf10-mediated current activity. The current of all four ion channels in SARS-CoV-2 was significantly inhibited by at least one of the applied compounds. Given the importance of these viroporins and others in related viruses<sup>14</sup>, this suggests that ion channel inhibitors might provide an attractive principle for future antiviral therapy. Future studies are required to determine the dose-response characteristics of the SARS-CoV-2 viroporins for the tested inhibitors. The ion selectivity of the new viroporins also needs to be investigated further, especially considering the continued threat of future coronavirus pandemics.

Amantadine (and analogues) has for many years been employed in the clinic for the treatment of influenza A virus infection and for the treatment of dyskinesia associated with Parkinson's' disease. In Influenza A, amantadine inhibits the M2 ion channel, and in parkinsonism, it blocks the NMDA-type glutamate receptor, thereby increasing dopamine levels. A very recent questionnaire-based study on 22 patients with neurological diseases also treated with amantadine or the related memantine, suggested a protective role of these drugs against COVID-19 manifestations<sup>15</sup>. The use of amantadine rests on several decades of clinical experience, facilitating a potential repurposing of amantadine for the prevention and treatment of SARS-CoV-2 infection and pathogenesis. Moreover, the manufacture of amantadine is uncomplicated and cheap and a distribution system is already in place, making the drug readily available for the global community. Globally, more than 3000 clinical investigations (clinicaltrials.gov) have been initiated within just a few months, testing a wide variety of approaches to prevent, treat, relieve, and diagnose SARS-CoV-2 infection. While this is indeed impressive and innovative from a scientific, developmental, and medical point of view, it also illustrates an unprecedented open and collaborative approach by regulatory authorities worldwide – underlining the enormous and urgent medical need. At present, amantadine is not included in any of these studies. We propose that amantadine could be an efficient treatment of COVID-19, possibly in combination with other antivirals and/or anti-inflammatory drugs, warranting testing for this purpose, better today than tomorrow.

## Materials And Methods

*RNA preparation and heterologous expression in Xenopus laevis oocytes* - The viroporin gene constructs where cloned into the pXOOM vector<sup>18</sup> between the BamHI and NotI restriction sites with a Kozak sequence (5'-ACCATG-3', initiator ATG underlined) following the BamHI site. Gene synthesis and cloning was performed at GenScript (USA). The plasmids were transformed into *E. coli* TOP10 cells according to the manual. Cells were plated on LB-agar plates with 50 µg/mL Kanamycin and incubated at 37 °C overnight. A single colony from the plate was used to inoculate 75 mL of LB broth containing 50 µg/mL Kanamycin in a 250 mL glass baffled shake flask in a shaking incubator (250 rpm) overnight at 37 °C. Fifty mL of the overnight culture was Midiprepped (GenElute™ HP Plasmid Midiprep Kit - Sigma) according to the manual. The concentration of the isolated plasmids was determined by absorbance ( $A_{260\text{ nm}}$ ). The plasmids were all precipitated with isopropanol according to the instructions in the Midiprep manual and dissolved in a smaller volume and stored at -80 °C. The plasmids were linearized downstream from the poly-A segment with XbaI and purified with the High Pure PCR Product Purification Kit (Roche) and their concentration determined by absorbance ( $A_{260\text{ nm}}$ ). The linearized plasmids were *in vitro* transcribed using T7 mMessage machine according to manufacturer's instructions (Ambion, Austin, TX). mRNA was extracted with MEGAclear (Ambion, Austin, TX). The concentration of the purified mRNA was determined by absorbance ( $A_{260\text{ nm}}$ ) and microinjected into defolliculated *Xenopus laevis* oocytes. Oocytes were either purchased from Ecocyte Bioscience, Germany, or surgically removed (in house) from the *Xenopus laevis* frogs and processed as described previously<sup>19</sup>. Animal handling was performed

under a license from the Danish Ministry of Justice and in agreement with the European Community guidelines for the use of experimental animals. Oocytes were kept in Kulori medium (in mM): 90 NaCl, 1 KCl, 1 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 5 HEPES (pH 7.4 adjusted with 2M tris-base ((HOCH<sub>2</sub>)<sub>3</sub>CNH<sub>2</sub>)) for 3-4 days at 19 °C prior to experiments.

*Electrophysiology in viroporin-expressing oocytes* - Oocyte electrophysiology was performed with two-electrode voltage clamp (TEVC) at room temperature using borosilicate glass capillary electrodes with a resistance of 1-3 MΩ when filled with 1 M KCl. The conductance was measured using the pClamp 9.2 or 10.4 software (Axon Instruments, Molecular Devices, San Jose, US) together with the Clampator One amplifier (model CA-1B, Dagan Co., Minneapolis, US) and the A/D converter Digidata 1440A (Molecular Devices, San Jose, US). The currents were low-pass filtered at 500 Hz and sampled at 1 kHz. All current measurements were derived from a 13-step voltage clamp protocol (200 ms, 15 mV increments from -130 mV to +50 mV) with a holding potential of -20 mV. The control solution contained the following in mM: 100 NaCl, 2 KCl, 1 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 HEPES, adjusted to a pH of 7.4 with 2 M tris-base ((HOCH<sub>2</sub>)<sub>3</sub>CNH<sub>2</sub>). In experiments employing inhibitors, the oocytes were locally perfused via the recording chamber prior to and during recordings. All inhibitors were purchased from Sigma and dissolved either in MilliQ (Amantadine, CAS no 665-66-7; Pyronin B, CAS no 2150-48-3; Pyronin Y, CAS no 92-32-0) or DMSO (Rimantadine, CAS no 1501-84-4; Adamantane CAS no 281-23-2; Emodin, CAS no 518-82-1; Xanthene CAS no 92-83-1) and diluted in control solution to a final test concentration of 10 μM. The individual drugs were diluted in MilliQ or DMSO at the day of the experiments, and the corresponding vehicle controls were applied. When applying inhibitors dissolved in DMSO, the same concentration was added to the control solution). Original stocks were stored at room temperature (kept dark). Employed working solutions were discarded after each experimentation.

## Declarations

### Competing interests:

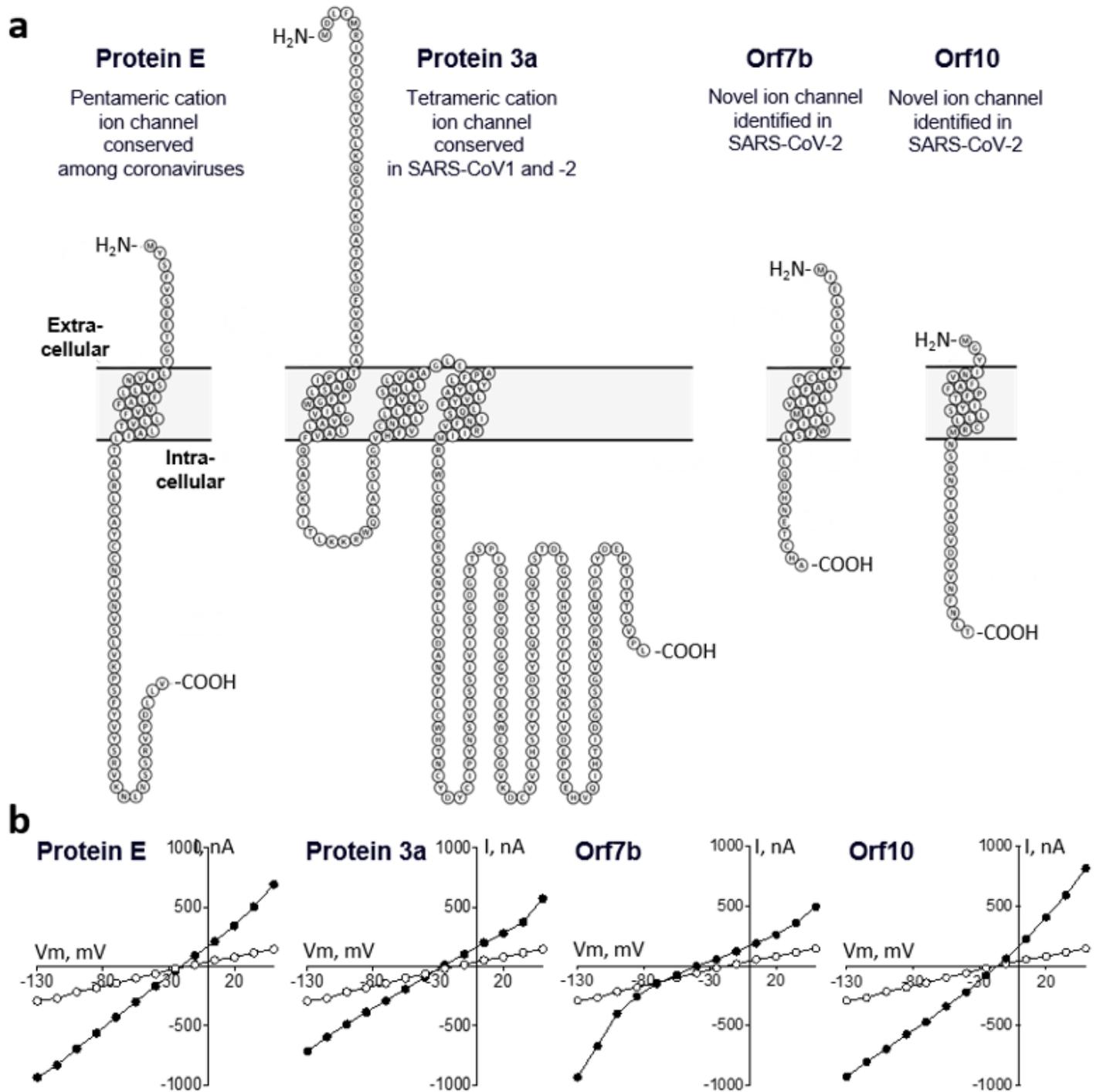
The authors declare no competing interests.

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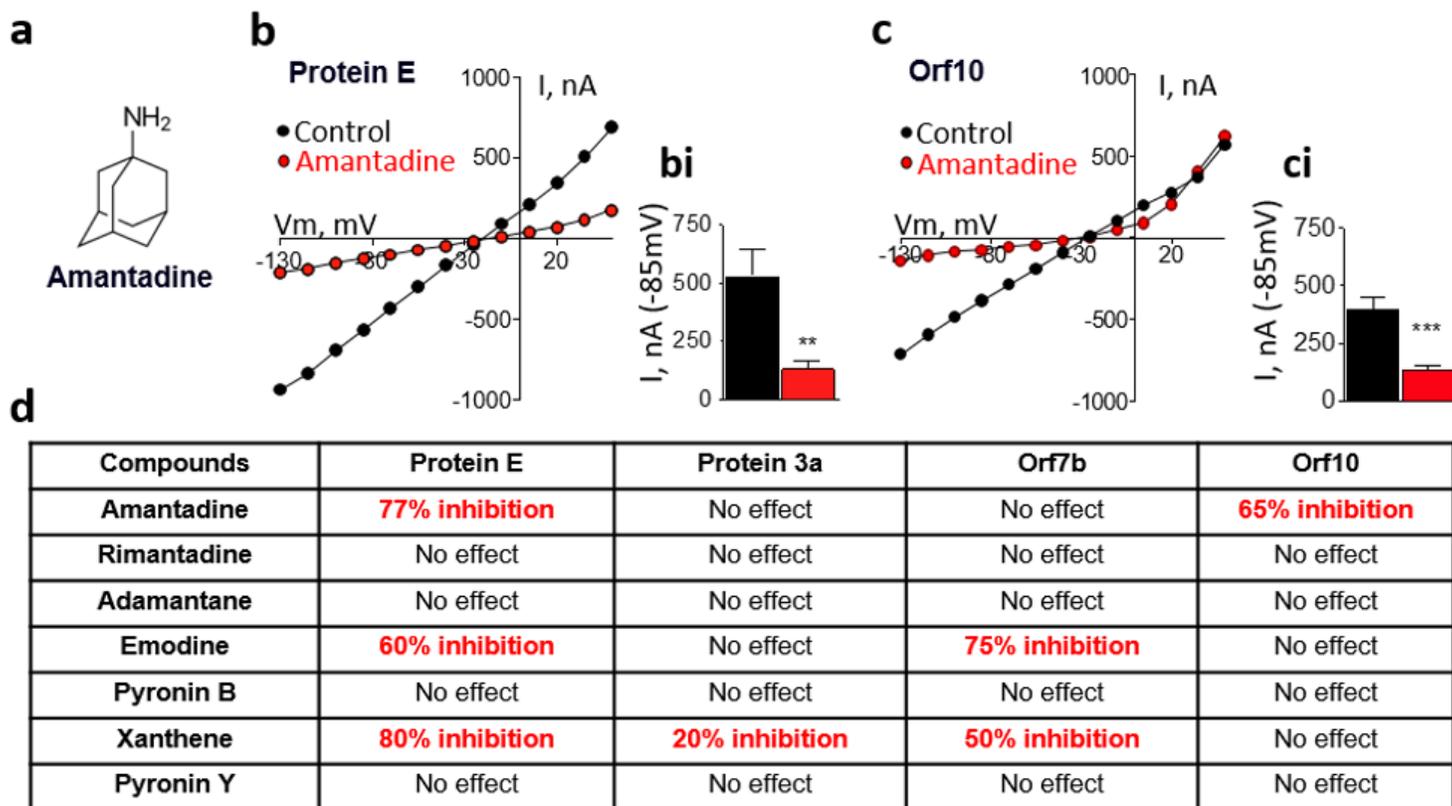
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## Figures



**Figure 1**

Overview of four viroporins expressed in SARS-CoV-2: Protein E, Protein 3a, Orf7b and Orf10. a, Overall structure and ion channel function of known (Protein E and -3a) and novel viroporins identified in current study (Orf7b and -10). The membrane topology was predicted by TMHMM2 16 and displayed using Protter 17. b, Ion channel activity, determined in *Xenopus laevis* oocytes with conventional two-electrode voltage clamp effect of seven selected drugs on the known (Protein E and 3a) and novel (Orf7b and -10) viroporins (n = 9-10 oocytes).



**Figure 2**

Several drugs inhibit the activity of the four ion channels from SARS-CoV-2; amantadine is among the most efficacious. Structure of amantadine (a) together with its inhibition of the ion channel activity of Protein E (b) and Orf10 ion (c), determined in *Xenopus laevis* oocytes with conventional two-electrode voltage clamp. Representative I/V curves of Protein E and Orf10-mediated activity in control solution without amantadine (shown in black) or with amantadine (shown in red). bi and ci, Current activity, I, at -85 mV obtained in control solution or after exposure to amantadine is shown as bars. Black bars correspond to current activity without drug, and red bars to the response with amantadine present. \*\* =  $p < 0.01$ . \*\*\* =  $p < 0.001$ ; Student's t-test,  $n = 9-10$  oocytes. d, Table summarizing the effect of seven selected compounds on the known (Protein E and -3a) and the herein identified (Orf7b and -10) ion channel activity, determined in *Xenopus laevis* oocyte with conventional two-electrode voltage clamp ( $n = 9-10$  oocytes).