

# Journal Pre-proof

The FDA-approved Drug Ivermectin inhibits the replication of SARS-CoV-2 *in vitro*

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1 **The FDA-approved Drug Ivermectin inhibits the replication of SARS-CoV-2 *in vitro*.**

2

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16 **Summary**

17 Although several clinical trials are now underway to test possible therapies, the worldwide  
18 response to the COVID-19 outbreak has been largely limited to monitoring/containment. We  
19 report here that Ivermectin, an FDA-approved anti-parasitic previously shown to have broad-  
20 spectrum anti-viral activity *in vitro*, is an inhibitor of the causative virus (SARS-CoV-2), with  
21 a single addition to Vero-hSLAM cells 2 hours post infection with SARS-CoV-2 able to  
22 effect ~5000-fold reduction in viral RNA at 48 h. Ivermectin therefore warrants further  
23 investigation for possible benefits in humans.

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27 Ivermectin is an FDA-approved broad spectrum anti-parasitic agent<sup>1</sup> that in recent years we,  
28 along with other groups, have shown to have anti-viral activity against a broad range of  
29 viruses<sup>2-5</sup> *in vitro*. Originally identified as an inhibitor of interaction between the human  
30 immunodeficiency virus-1 (HIV-1) integrase protein (IN) and the importin (IMP)  $\alpha/\beta$ 1  
31 heterodimer responsible for IN nuclear import<sup>6</sup>, Ivermectin has since been confirmed to  
32 inhibit IN nuclear import and HIV-1 replication<sup>5</sup>. Other actions of ivermectin have been  
33 reported<sup>7</sup>, but ivermectin has been shown to inhibit nuclear import of host (eg. <sup>8, 9</sup>) and viral  
34 proteins, including simian virus SV40 large tumour antigen (T-ag) and dengue virus (DENV)  
35 non-structural protein 5<sup>5, 6</sup>. Importantly, it has been demonstrated to limit infection by RNA  
36 viruses such as DENV 1-4<sup>4</sup>, West Nile Virus<sup>10</sup>, Venezuelan equine encephalitis virus  
37 (VEEV)<sup>3</sup> and influenza<sup>2</sup>, with this broad spectrum activity believed to be due to the reliance  
38 by many different RNA viruses on IMP $\alpha/\beta$ 1 during infection<sup>11, 12</sup>. Ivermectin has similarly  
39 been shown to be effective against the DNA virus pseudorabies virus (PRV) both *in vitro* and  
40 *in vivo*, with ivermectin treatment shown to increase survival in PRV-infected mice<sup>13</sup>.  
41 Efficacy was not observed for ivermectin against Zika virus (ZIKV) in mice, but the authors  
42 acknowledged that study limitations justified re-evaluation of ivermectin's anti-ZIKV  
43 activity<sup>14</sup>. Finally, ivermectin was the focus of a phase III clinical trial in Thailand in 2014-  
44 2017, against DENV infection, in which a single daily oral dose was observed to be safe and  
45 resulted in a significant reduction in serum levels of viral NS1 protein, but no change in  
46 viremia or clinical benefit was observed (see below)<sup>15</sup>.

47 The causative agent of the current COVID-19 pandemic, SARS-CoV-2, is a single  
48 stranded positive sense RNA virus that is closely related to severe acute respiratory syndrome  
49 coronavirus (SARS-CoV). Studies on SARS-CoV proteins have revealed a potential role for  
50 IMP $\alpha/\beta$ 1 during infection in signal-dependent nucleocytoplasmic shuttling of the SARS-CoV  
51 Nucleocapsid protein<sup>16-18</sup>, that may impact host cell division<sup>19, 20</sup>. In addition, the SARS-CoV

52 accessory protein ORF6 has been shown to antagonize the antiviral activity of the STAT1  
53 transcription factor by sequestering IMP $\alpha$ / $\beta$ 1 on the rough ER/Golgi membrane<sup>21</sup>. Taken  
54 together, these reports suggested that ivermectin's nuclear transport inhibitory activity may  
55 be effective against SARS-CoV-2.

56 To test the antiviral activity of ivermectin towards SARS-CoV-2, we infected  
57 Vero/hSLAM cells with SARS-CoV-2 isolate Australia/VIC01/2020 at an MOI of 0.1 for 2  
58 h, followed by the addition of 5  $\mu$ M ivermectin. Supernatant and cell pellets were harvested  
59 at days 0-3 and analysed by RT-PCR for the replication of SARS-CoV-2 RNA (**Fig. 1A/B**).  
60 At 24 h, there was a 93% reduction in viral RNA present in the supernatant (indicative of  
61 released virions) of samples treated with ivermectin compared to the vehicle DMSO.  
62 Similarly a 99.8% reduction in cell-associated viral RNA (indicative of unreleased and  
63 unpackaged virions) was observed with ivermectin treatment. By 48h this effect increased to  
64 an ~5000-fold reduction of viral RNA in ivermectin-treated compared to control samples,  
65 indicating that ivermectin treatment resulted in the effective loss of essentially all viral  
66 material by 48 h. Consistent with this idea, no further reduction in viral RNA was observed at  
67 72 h. As we have observed previously<sup>3-5</sup>, no toxicity of ivermectin was observed at any of the  
68 timepoints tested, in either the sample wells or in parallel tested drug alone samples.

69 To further determine the effectiveness of ivermectin, cells infected with SARS-CoV-2 were  
70 treated with serial dilutions of ivermectin 2 h post infection and supernatant and cell pellets  
71 collected for real-time RT-PCR at 48 h (**Fig. 1C/D**). As above, a >5000 reduction in viral  
72 RNA was observed in both supernatant and cell pellets from samples treated with 5  $\mu$ M  
73 ivermectin at 48 h, equating to a 99.98% reduction in viral RNA in these samples. Again, no  
74 toxicity was observed with ivermectin at any of the concentrations tested. The IC<sub>50</sub> of  
75 ivermectin treatment was determined to be ~2 $\mu$ M under these conditions. Underlining the  
76 fact that the assay indeed specifically detected SARS-CoV-2, RT-PCR experiments were

77 repeated using primers specific for the viral RdRp gene (**Fig. 1E/F**) rather than the E gene  
78 (above), with nearly identical results observed for both released (supernatant) and cell-  
79 associated virus.

80 Taken together these results demonstrate that ivermectin has antiviral action against  
81 the SARS-CoV-2 clinical isolate *in vitro*, with a single dose able to control viral replication  
82 within 24-48 h in our system. We hypothesise that this is likely through inhibiting IMP $\alpha$ / $\beta$ 1-  
83 mediated nuclear import of viral proteins (**Fig. 1G**), as shown for other RNA viruses<sup>4, 5, 10</sup>;  
84 confirmation of this mechanism in the case of SARS-CoV-2, and identification of the specific  
85 SARS-CoV-2 and/or host component(s) impacted (see<sup>10</sup>) is an important focus future work  
86 in this laboratory. Ultimately, development of an effective anti-viral for SARS-CoV-2, if  
87 given to patients early in infection, could help to limit the viral load, prevent severe disease  
88 progression and limit person-person transmission. Benchmarking testing of ivermectin  
89 against other potential antivirals for SARS-CoV-2 with alternative mechanisms of action<sup>22-26</sup>  
90 would thus be important as soon as practicable. This Brief Report raises the possibility that  
91 ivermectin could be a useful antiviral to limit SARS-CoV-2, in similar fashion to those  
92 already reported<sup>22-26</sup>; until one of these is proven to be beneficial in a clinical setting, all  
93 should be pursued as rapidly as possible.

94 Ivermectin has an established safety profile for human use<sup>1, 12, 27</sup>, and is FDA-  
95 approved for a number of parasitic infections<sup>1, 27</sup>. Importantly, recent reviews and meta-  
96 analysis indicate that high dose ivermectin has comparable safety as the standard low-dose  
97 treatment, although there is not enough evidence to make conclusions about the safety profile  
98 in pregnancy<sup>28, 29</sup>. The critical next step in further evaluation for possible benefit in COVID-  
99 19 patients will be to examine a multiple addition dosing regimen that mimics the current  
100 approved usage of ivermectin in humans. As noted, ivermectin was the focus of a recent  
101 phase III clinical trial in dengue patients in Thailand, in which a single daily dose was found

102 to be safe but did not produce any clinical benefit. However, the investigators noted that an  
103 improved dosing regimen might be developed, based on pharmacokinetic data<sup>15</sup>. Although  
104 DENV is clearly very different to SARS-CoV-2, this trial design should inform future work  
105 going forward. Altogether the current report, combined with a known-safety profile,  
106 demonstrates that ivermectin is worthy of further consideration as a possible SARS-CoV-2  
107 antiviral.

108

## 109 **Methods**

### 110 *Cell culture, viral infection and drug treatment*

111 Vero/hSLAM cells<sup>30</sup> were maintained in Earle's Minimum Essential Medium (EMEM)  
112 containing 7% Fetal Bovine Serum (FBS) (Bovogen Biologicals, Keilor East, AUS) 2 mM L-  
113 Glutamine, 1 mM Sodium pyruvate, 1500 mg/L sodium bicarbonate, 15 mM HEPES and 0.4  
114 mg/ml geneticin at 37°C, 5% CO<sub>2</sub>. Cells were seeded into 12-well tissue culture plates 24 h  
115 prior to infection with SARS-CoV-2 (Australia/VIC01/2020 isolate) at an MOI of 0.1 in  
116 infection media (as per maintenance media but containing only 2% FBS) for 2 h. Media  
117 containing inoculum was removed and replaced with 1 mL fresh media (2% FBS) containing  
118 Ivermectin at the indicated concentrations or DMSO alone and incubated as indicated for 0-3  
119 days. At the appropriate timepoint, cell supernatant was collected and spun for 10 min at  
120 6,000g to remove debris and the supernatant transferred to fresh collection tubes. The cell  
121 monolayers were collected by scraping and resuspension into 1 mL fresh media (2% FBS).  
122 Toxicity controls were set up in parallel in every experiment on uninfected cells.

123

### 124 *Generation of SARS-CoV-2 cDNA*

125 RNA was extracted from 200 µL aliquots of sample supernatant or cell suspension using the  
126 QIAamp 96 Virus QIAcube HT Kit (Qiagen, Hilden, Germany) and eluted in 60 µl. Reverse

127 transcription was performed using the BioLine SensiFAST cDNA kit (Bioline, London,  
128 United Kingdom), total reaction mixture (20 µl), containing 10 µL of RNA extract, 4 µl of 5x  
129 TransAmp buffer, 1µl of Reverse Transcriptase and 5 µl of Nuclease free water. The  
130 reactions were incubated at 25°C for 10 min, 42°C for 15 min and 85°C for 5 min.

131

132 ***Detection of SARS-CoV-2 using a TaqMan Real-time RT-PCR assay.***

133 TaqMan RT-PCR assay were performed using 2.5 µl cDNA, 10 µl Primer Design  
134 PrecisionPLUS qPCR Master Mix 1 µM Forward (5'- AAA TTC TAT GGT GGT TGG CAC  
135 AAC ATG TT-3'), 1 µM Reverse (5'- TAG GCA TAG CTC TRT CAC AYT T-3') primers  
136 and 0.2 µM probe (5'-FAM- TGG GTT GGG ATT ATC-MGBNFQ-3') targeting the  
137 BetaCoV RdRp (RNA-dependent RNA polymerase) gene or Forward (5'-ACA GGT ACG  
138 TTA ATA GTT AAT AGC GT -3'), 1 µM Reverse (5'-ATA TTG CAG CAG TAC GCA  
139 CAC A-3') primers and 0.2 µM probe (5'-FAM-ACA CTA GCC ATC CTT ACT GCG CTT  
140 CG-

141 286 NFQ-3') targeting the BetaCoV E-gene<sup>31</sup>. Real-time RT-PCR assays were performed on  
142 an Applied Biosystems ABI 7500 Fast real-time PCR machine (Applied Biosystems, Foster  
143 City, CA, USA) using cycling conditions of 95°C for 2 min, 95°C for 5 s, 60°C for 24 s.  
144 SARS-CoV-2 cDNA (Ct~28) was used as a positive control. Calculated Ct values were  
145 converted to fold-reduction of treated samples compared to control using the  $\Delta C_t$  method  
146 (fold changed in viral RNA =  $2^{\Delta C_t}$ ) and expressed as % of DMSO alone sample. IC50  
147 values were fitted using 3 parameter dose response curves in GraphPad prism.

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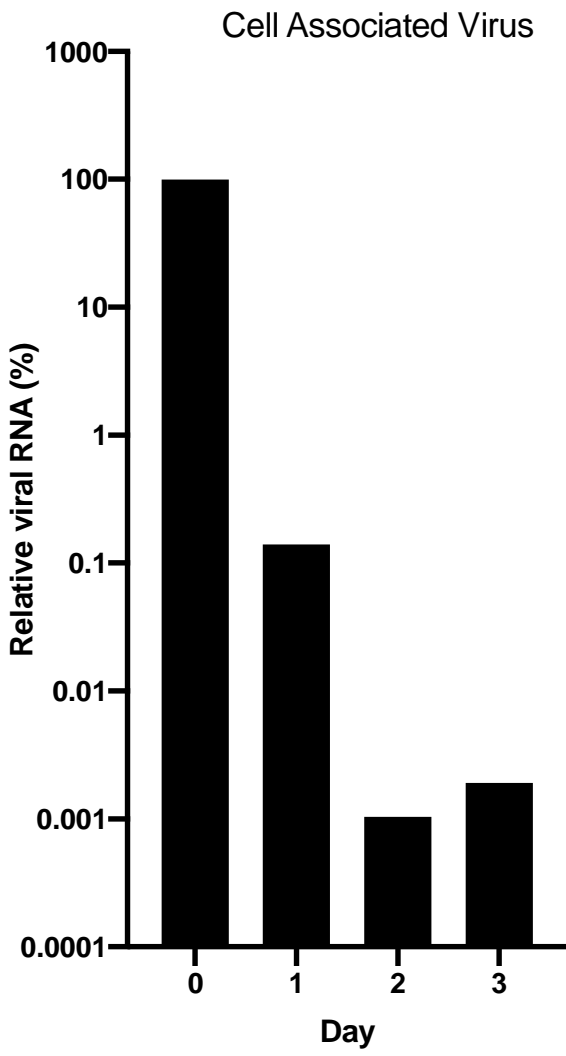
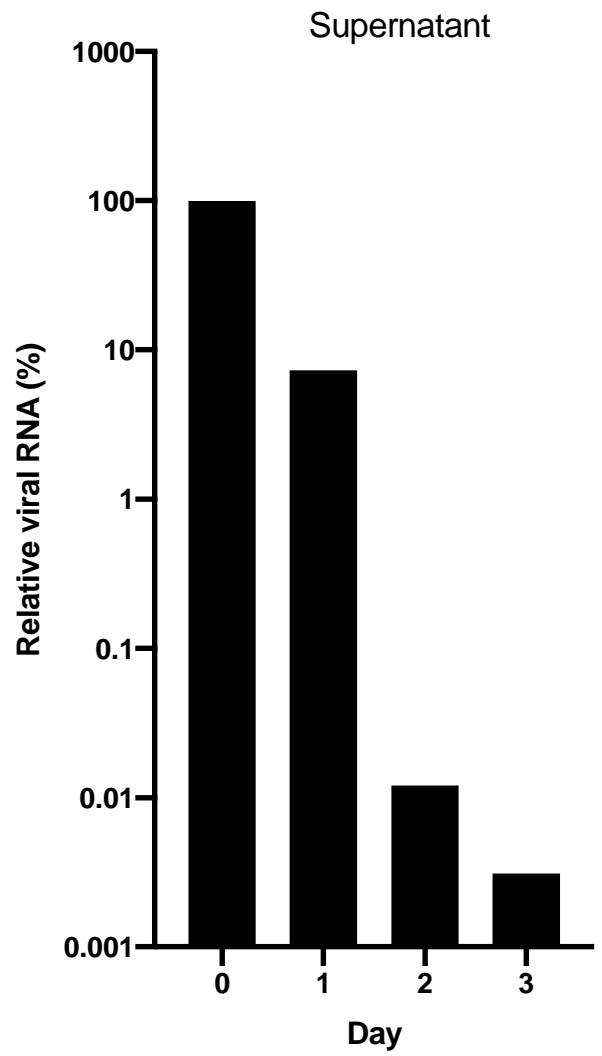
157 **References**

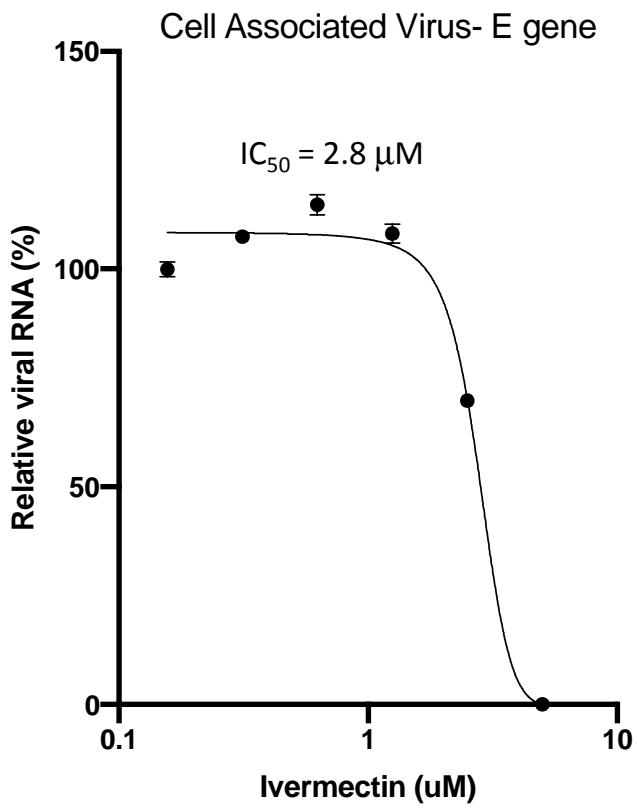
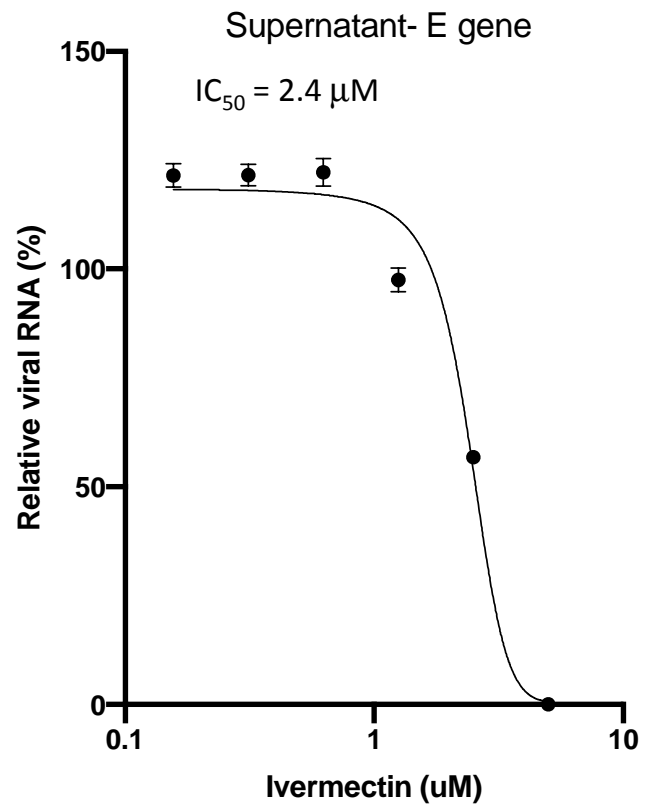
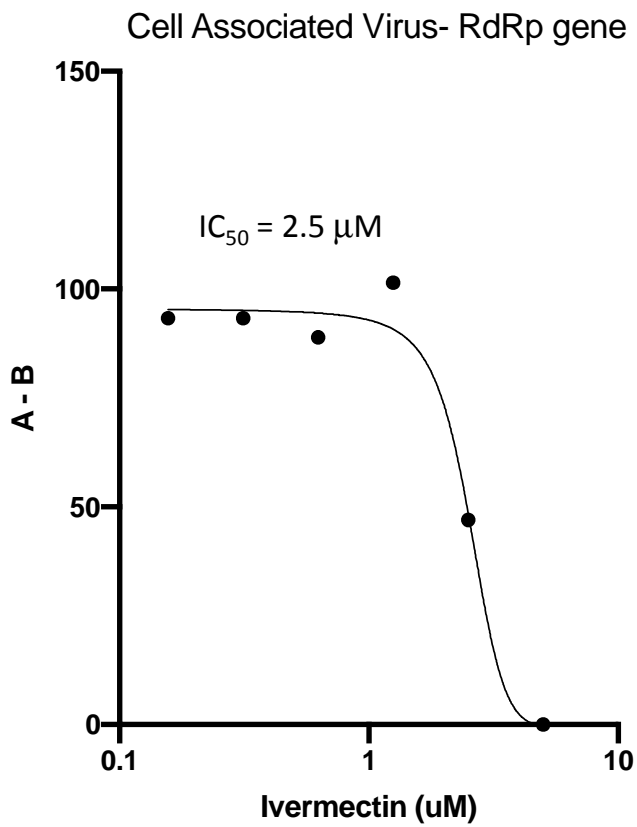
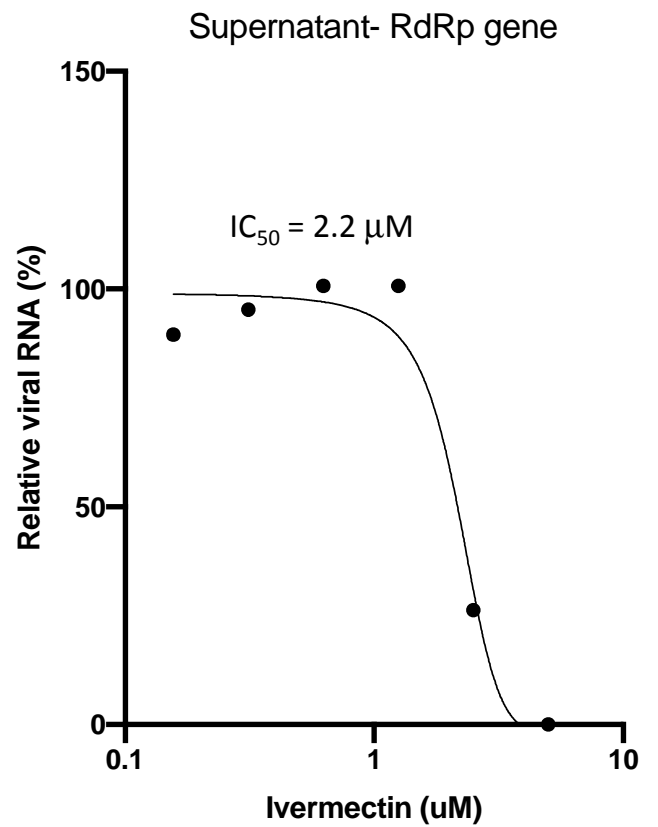
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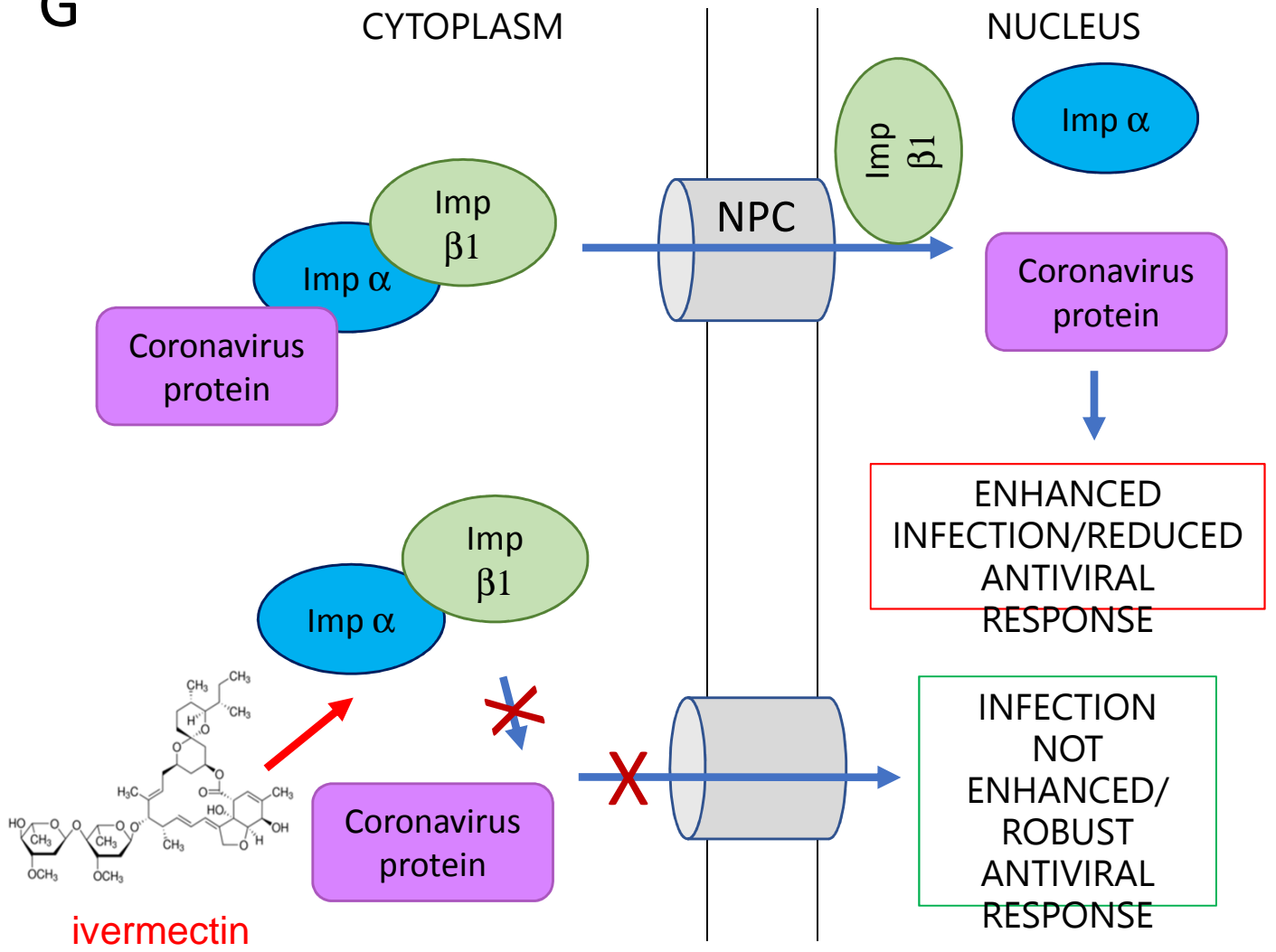
237 **Figure 1: Ivermectin is a potent inhibitor of the SARS-CoV-2 clinical isolate**  
238 **Australia/VIC01/2020.** Vero/hSLAM cells were infected with SARS-CoV-2 clinical  
239 isolate Australia/VIC01/2020 (MOI = 0.1) for 2 h prior to addition of vehicle (DMSO) or  
240 Ivermectin at the indicated concentrations. Samples were taken at 0-3 days post infection for  
241 quantitation of viral load using real-time PCR of cell associated virus (**A**) or supernatant (**B**).  
242  $IC_{50}$  values were determined in subsequent experiments at 48 h post infection using the  
243 indicated concentrations of Ivermectin (treated at 2 h post infection as per **A/B**). Triplicate  
244 real-time PCR analysis was performed on cell associated virus (**C/E**) or supernatant (**D/F**)  
245 using probes against either the SARS-CoV-2 E (**C/D**) or RdRp (**E/F**) genes. Results represent  
246 mean  $\pm$  SD (n=3). 3 parameter dose response curves were fitted using GraphPad prism to  
247 determine  $IC_{50}$  values (indicated). **G.** Schematic of ivermectin's proposed antiviral action on  
248 coronavirus.  $IMP\alpha/\beta 1$  binds to the coronavirus cargo protein in the cytoplasm (top) and  
249 translocates it through the nuclear pore complex (NPC) into the nucleus where the complex  
250 falls apart and the viral cargo can reduce the host cell's antiviral response, leading to  
251 enhanced infection. Ivermectin binds to and destabilises the  $Imp\alpha/\beta 1$  heterodimer thereby  
252 preventing  $Imp\alpha/\beta 1$  from binding to the viral protein (bottom) and preventing it from  
253 entering the nucleus. This likely results in reduced inhibition of the antiviral responses,  
254 leading to a normal, more efficient antiviral response.

255

**A****B**

**C****D****E****F**

G



## Highlights

- Ivermectin is an inhibitor of the COVID-19 causative virus (SARS-CoV-2) *in vitro*.
- A single treatment able to effect ~5000-fold reduction in virus at 48h in cell culture.
- Ivermectin is FDA-approved for parasitic infections, and therefore has a potential for repurposing.
- Ivermectin is widely available, due to its inclusion on the WHO model list of essential medicines.

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