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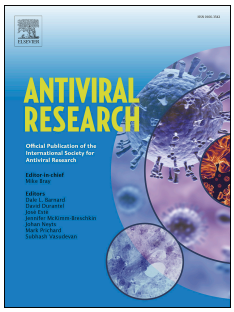

The FDA-approved Drug Ivermectin inhibits the replication of SARS-CoV-2 *in vitro*

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

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Summary

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

Ivermectin is an FDA-approved broad spectrum anti-parasitic agent[1] that in recent years we, along with other groups, have shown to have anti-viral activity against a broad range of viruses[2-5] *in vitro*. Originally identified as an inhibitor of interaction between the human immunodeficiency virus-1 (HIV-1) integrase protein (IN) and the importin (IMP) α/β1 heterodimer responsible for IN nuclear import[6], Ivermectin has since been confirmed to inhibit IN nuclear import and HIV-1 replication[5]. Other actions of ivermectin have been reported[7], but ivermectin has been shown to inhibit nuclear import of host (eg. [8, 9]) and viral proteins, including simian virus SV40 large tumour antigen (T-ag) and dengue virus (DENV) non-structural protein 5[5, 6]. Importantly, it has been demonstrated to limit infection by RNA viruses such as DENV 1-4[4], West Nile Virus[10], Venezuelan equine encephalitis virus (VEEV)[3] and influenza[2], with this broad spectrum activity believed to be due to the reliance by many different RNA viruses on IMPα/β1 during infection[11, 12]. Ivermectin has similarly been shown to be effective against the DNA virus pseudorabies virus (PRV) both *in vitro* and *in vivo*, with ivermectin treatment shown to increase survival in PRV-infected mice[13]. Efficacy was not observed for ivermectin against Zika virus (ZIKV) in mice, but the authors acknowledged that study limitations justified re-evaluation of ivermectin's anti-ZIKV activity[14]. Finally, ivermectin was the focus of a phase III clinical trial in Thailand in 2014-2017, against DENV infection, in which a single daily oral dose was observed to be safe and resulted in a significant reduction in serum levels of viral NS1 protein, but no change in viremia or clinical benefit was observed (see below)[15].

The causative agent of the current COVID-19 pandemic, SARS-CoV-2, is a single stranded positive sense RNA virus that is closely related to severe acute respiratory syndrome coronavirus (SARS-CoV). Studies on SARS-CoV proteins have revealed a potential role for IMPα/β1 during infection in signal-dependent nucleocytoplasmic shutting of the SARS-CoV Nucleocapsid protein[16-18], that may impact host cell division[19, 20]. In addition, the SARS-CoV

accessory protein ORF6 has been shown to antagonize the antiviral activity of the STAT1 transcription factor by sequestering IMPα/β1 on the rough ER/Golgi membrane[21]. Taken together, these reports suggested that ivermectin's nuclear transport inhibitory activity may be effective against SARS-CoV-2.

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

To further determine the effectiveness of ivemectin, cells infected with SARS-CoV-2 were treated with serial dilutions of ivermectin 2 h post infection and supernatant and cell pellets collected for real-time RT-PCR at 48 h (**Fig. 1C/D**). As above, a >5000 reduction in viral RNA was observed in both supernatant and cell pellets from samples treated with 5 μM ivermectin at 48 h, equating to a 99.98% reduction in viral RNA in these samples. Again, no toxicity was observed with ivermectin at any of the concentrations tested. The IC50 of ivermectin treatment was determined to be ~2μM under these conditions. Underlining the fact that the assay indeed specifically detected SARS-CoV-2, RT-PCR experiments were

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

Taken together these results demonstrate that ivermectin has antiviral action against the SARS-CoV-2 clinical isolate *in vitro*, with a single dose able to control viral replication within 24-48 h in our system. We hypothesise that this is likely through inhibiting IMPα/β1-mediated nuclear import of viral proteins (**Fig. 1G**), as shown for other RNA viruses [4, 5, 10]; confirmation of this mechanism in the case of SARS-CoV-2, and identification of the specific SARS-CoV-2 and/or host component(s) impacted (see [10]) is an important focus future work in this laboratory. Ultimately, development of an effective anti-viral for SARS-CoV-2, if given to patients early in infection, could help to limit the viral load, prevent severe disease progression and limit person-person transmission. Benchmarking testing of ivermectin against other potential antivirals for SARS-CoV-2 with alternative mechanisms of action[22-26] would thus be important as soon as practicable. This Brief Report raises the possibility that ivermectin could be a useful antiviral to limit SARS-CoV-2, in similar fashion to those already reported[22-26]; until one of these is proven to be beneficial in a clinical setting, all should be pursued as rapidly as possible.

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

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

Methods

Cell culture, viral infection and drug treatment

Vero/hSLAM cells[30] were maintained in Earle's Minimum Essential Medium (EMEM) containing 7% Fetal Bovine Serum (FBS) (Bovogen Biologicals, Keilor East, AUS) 2 mM L-Glutamine, 1 mM Sodium pyruvate, 1500 mg/L sodium bicarbonate, 15 mM HEPES and 0.4 mg/ml geneticin at 37°C, 5% CO_2. Cells were seeded into 12-well tissue culture plates 24 h prior to infection with SARS-CoV-2 (Australia/VIC01/2020 isolate) at an MOI of 0.1 in infection media (as per maintenance media but containing only 2% FBS) for 2 h. Media containing inoculum was removed and replaced with 1 mL fresh media (2% FBS) containing Ivermectin at the indicated concentrations or DMSO alone and incubated as indicated for 0-3 days. At the appropriate timepoint, cell supernatant was collected and spun for 10 min at 6,000g to remove debris and the supernatant transferred to fresh collection tubes. The cell monolayers were collected by scraping and resuspension into 1 mL fresh media (2% FBS). Toxicity controls were set up in parallel in every experiment on uninfected cells.

Generation of SARS-CoV-2 cDNA

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

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

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

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

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

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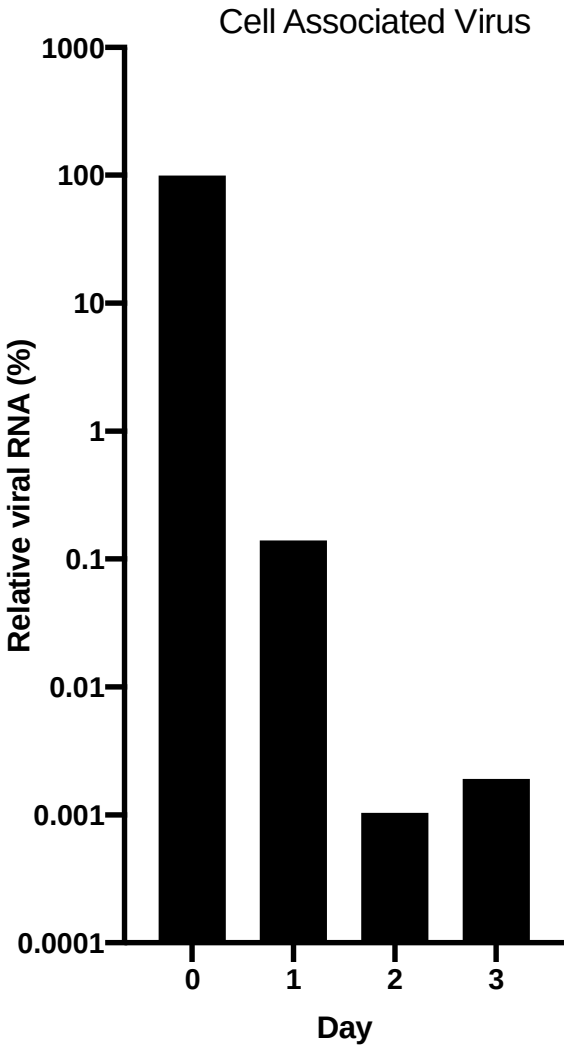
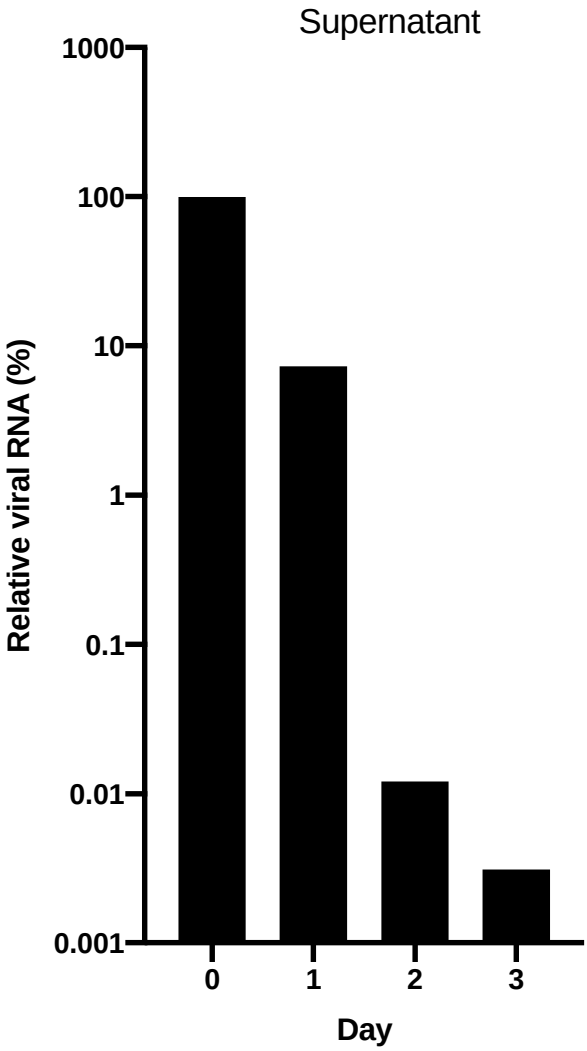
Figure 1: Ivermectin is a potent inhibitor of the SARS-CoV-2 clinical isolate Australia/VIC01/2020. Vero/hSLAM cells were in infected with SARS-CoV-2 clinical isolate Australia/VIC01/2020 (MOI = 0.1) for 2 h prior to addition of vehicle (DMSO) or Ivermectin at the indicated concentrations. Samples were taken at 0-3 days post infection for quantitation of viral load using real-time PCR of cell associated virus (**A**) or supernatant (**B**). IC_{50} values were determined in subsequent experiments at 48 h post infection using the indicated concentrations of Ivermectin (treated at 2 h post infection as per **A/B**). Triplicate real-time PCR analysis was performed on cell associated virus (**C/E**) or supernatant (**D/F**) using probes against either the SARS-CoV-2 E (**C/D**) or RdRp (**E/F**) genes. Results represent mean ± SD (n=3). 3 parameter dose response curves were fitted using GraphPad prism to determine IC_{50} values (indicated). **G.** Schematic of ivermectin's proposed antiviral action on coronavirus. IMPα/β1 binds to the coronavirus cargo protein in the cytoplasm (top) and translocates it through the nuclear pore complex (NPC) into the nucleus where the complex falls apart and the viral cargo can reduce the host cell's antiviral response, leading to enhanced infection. Ivermectin binds to and destabilises the Impα/β1 heterodimer thereby preventing Impα/β1 from binding to the viral protein (bottom) and preventing it from entering the nucleus. This likely results in reduced inhibition of the antiviral responses, leading to a normal, more efficient antiviral response.

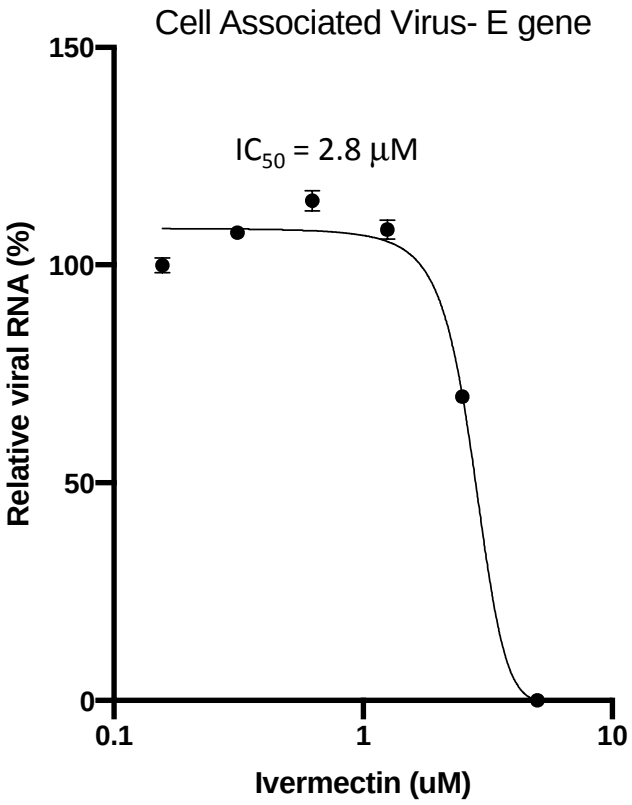
A


Cell Associated Virus
1000
100
10
1
0.1
0.01
0.001
0.0001
Relative viral RNA (%)
0
1
2
3
Day

B


Supernatant
1000
100
10
1
0.1
0.01
0.001
Relative viral RNA (%)
0
1
2
3
Day

C


Cell Associated Virus- E gene
IC50 = 2.8 µM
Relative viral RNA (%)
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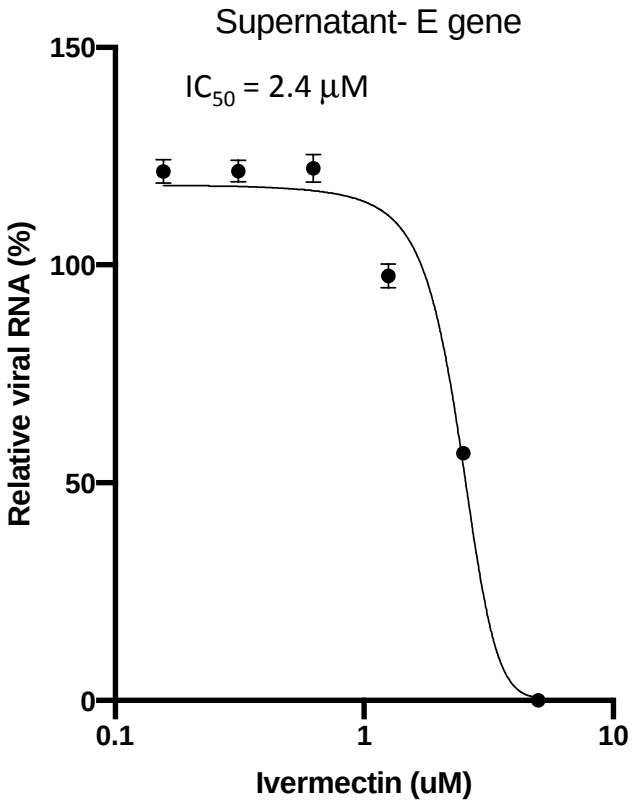
100
50
0
0.1
1
10
Ivermectin (uM)

D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IC50 = 2.4 µM
Relative viral RNA (%)
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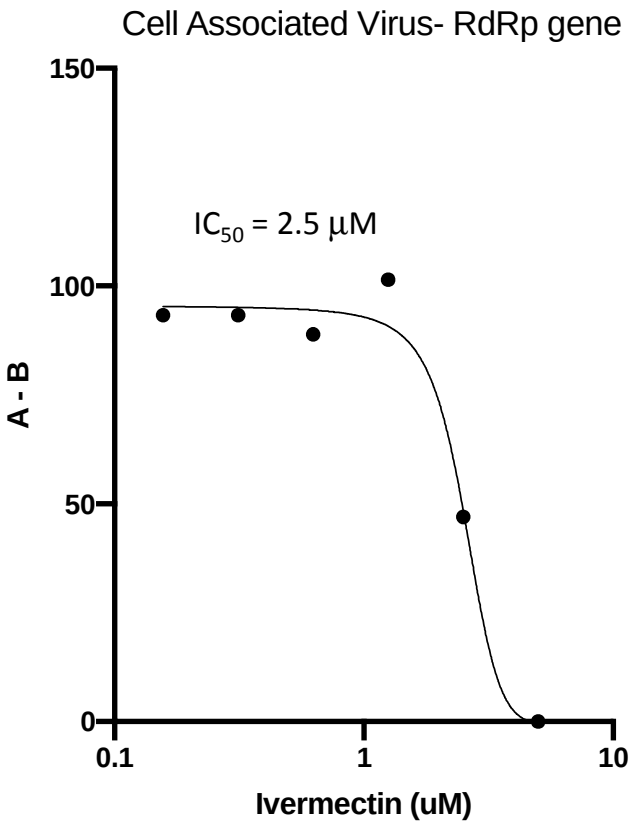
100
50
0
0.1
1
10
Ivermectin (uM)

E


Cell Associated Virus- RdRp gene
IC50 = 2.5 µM
A - B
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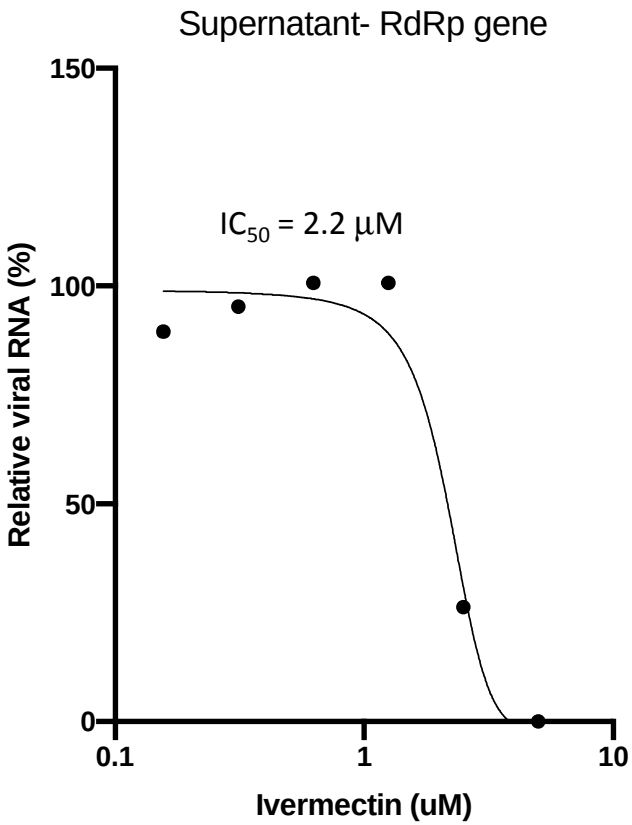
100
50
0
0.1
1
10
Ivermectin (uM)

F


Supernatant- RdRp gene
IC50 = 2.2 µM
Relative viral RNA (%)
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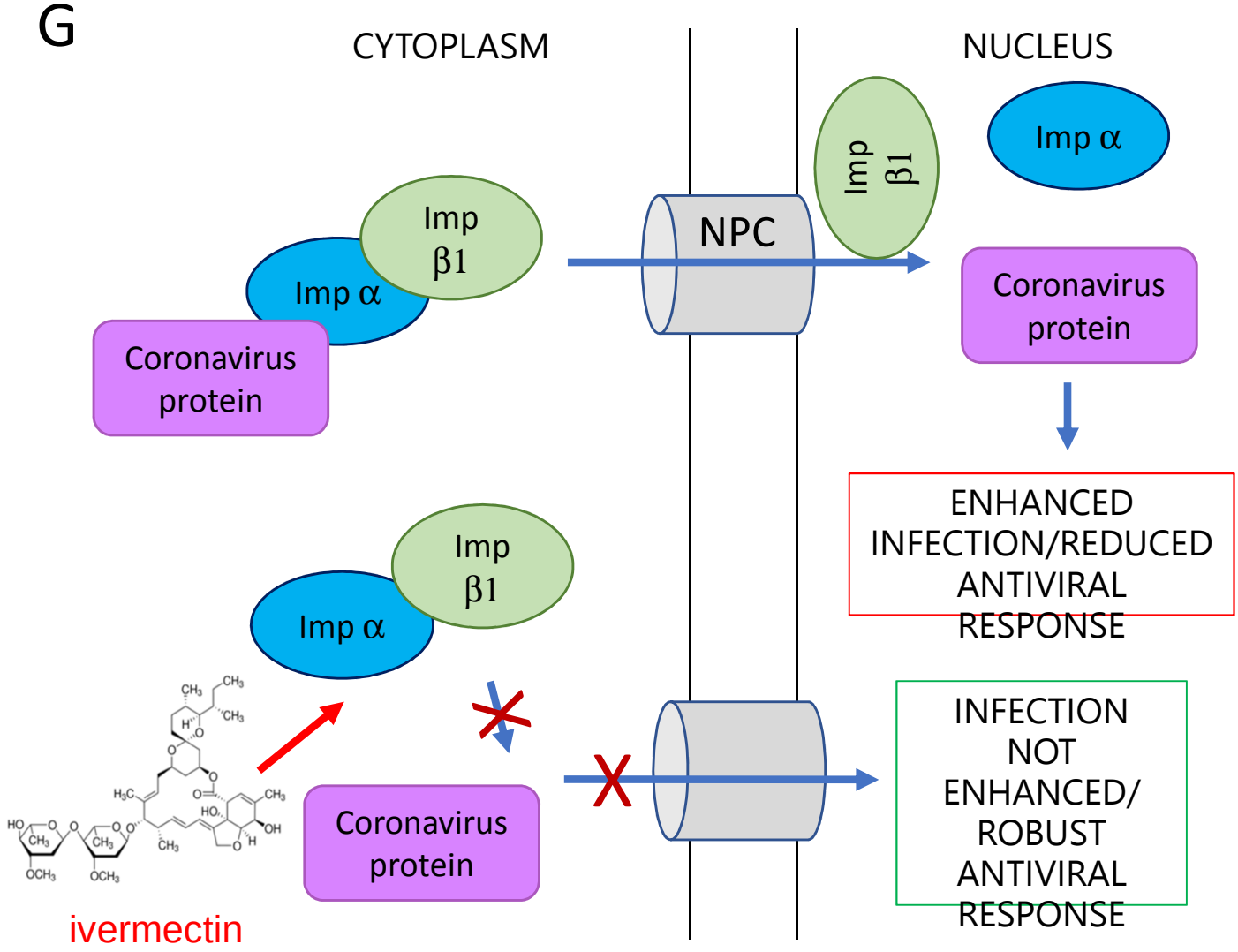
100
50
0
0.1
1
10
Ivermectin (uM)

G


CYTOPLASM
NUCLEUS
Imp β1
Imp α
NPC
Imp β1
Imp α
Coronavirus protein
Coronavirus protein
ENHANCED INFECTION/REDUCED ANTIVIRAL RESPONSE
Imp β1
Imp α
Coronavirus protein
INFECTION NOT ENHANCED/ ROBUST ANTIVIRAL RESPONSE
ivermectin

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.