Journal Pre-proof

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

Leon Caly, Julian D. Druce, Mike G. Catton, David A. Jans, Kylie M. Wagstaff

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

2

3   Leon Caly[1], Julian D. Druce[1], Mike G. Catton[1], David A. Jans[2] and Kylie M. Wagstaff[2*]

4   [1] *Victorian Infectious Diseases Reference Laboratory, Royal Melbourne Hospital, at the*

5   *Peter Doherty Institute for Infection and Immunity, Victoria, 3000, Australia.*

6   [2]*Biomedicine Discovery Institute, Monash University, Clayton, Vic, 3800 Australia*

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8   *** Corresponding author:**

9   Dr. Kylie Wagstaff, Biomedicine Discovery Institute, Monash University, Clayton, Vic, 3800

10   Australia

11   Tel: +61 3 9902 9348

12   Email: kylie.wagstaff@monash.edu

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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[1] that in recent years we,

28 along with other groups, have shown to have anti-viral activity against a broad range of

29 viruses[2-5] *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) α/β1

31 heterodimer responsible for IN nuclear import[6], Ivermectin has since been confirmed to

32 inhibit IN nuclear import and HIV-1 replication[5]. Other actions of ivermectin have been

33 reported[7], but ivermectin has been shown to inhibit nuclear import of host (eg. [8, 9]) and viral

34 proteins, including simian virus SV40 large tumour antigen (T-ag) and dengue virus (DENV)

35 non-structural protein 5[5, 6]. Importantly, it has been demonstrated to limit infection by RNA

36 viruses such as DENV 1-4[4], West Nile Virus[10], Venezuelan equine encephalitis virus

37 (VEEV)[3] and influenza[2], with this broad spectrum activity believed to be due to the reliance

38 by many different RNA viruses on IMPα/β1 during infection[11, 12]. 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[13].

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[14]. 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)[15].

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 IMPα/β1 during infection in signal-dependent nucleocytoplasmic shutting of the SARS-CoV

51 Nucleocapsid protein[16-18], that may impact host cell division[19, 20]. 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 IMPα/β1 on the rough ER/Golgi membrane[21]. 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 μM 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[3-5], 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 ivemectin, 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 μM

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 IC50 of

75   ivermectin treatment was determined to be ~2μM 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 IMPα/β1-

83   mediated nuclear import of viral proteins (**Fig. 1G**), as shown for other RNA viruses [4, 5, 10];

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 [10]) 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[22-26]

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[22-26]; 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[1, 12, 27], and is FDA-

95   approved for a number of parasitic infections[1, 27]. 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 [28, 29]. 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[15]. 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[30] 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_2. 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   PrecisonPLUS 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[31]. 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 ΔCt method

146   (fold changed in viral RNA = $2^{\Delta Ct}$) 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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152 *Funding*

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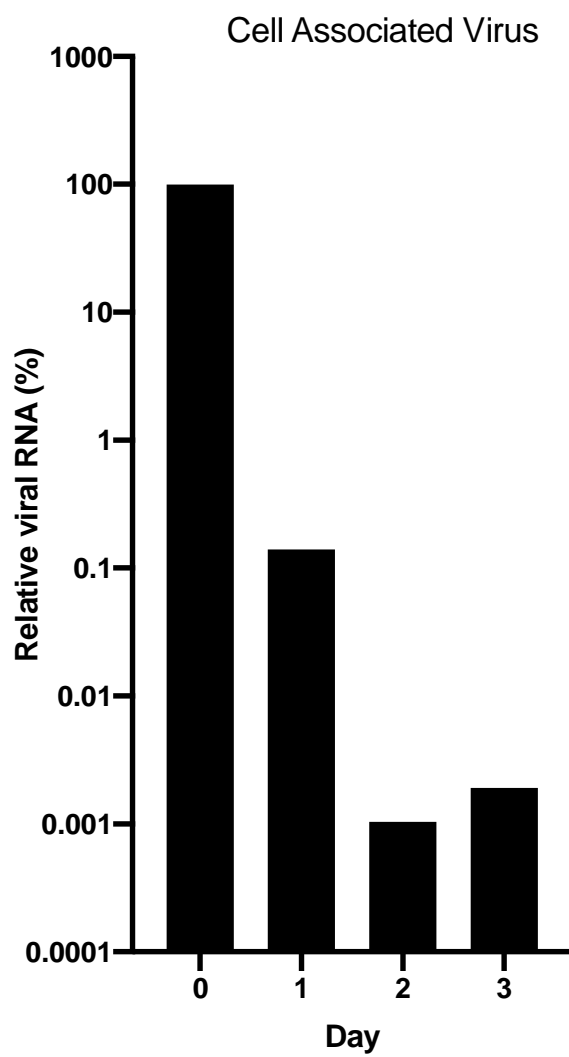154 for KMW and an NHMRC SPRF (APP1103050) for DAJ.

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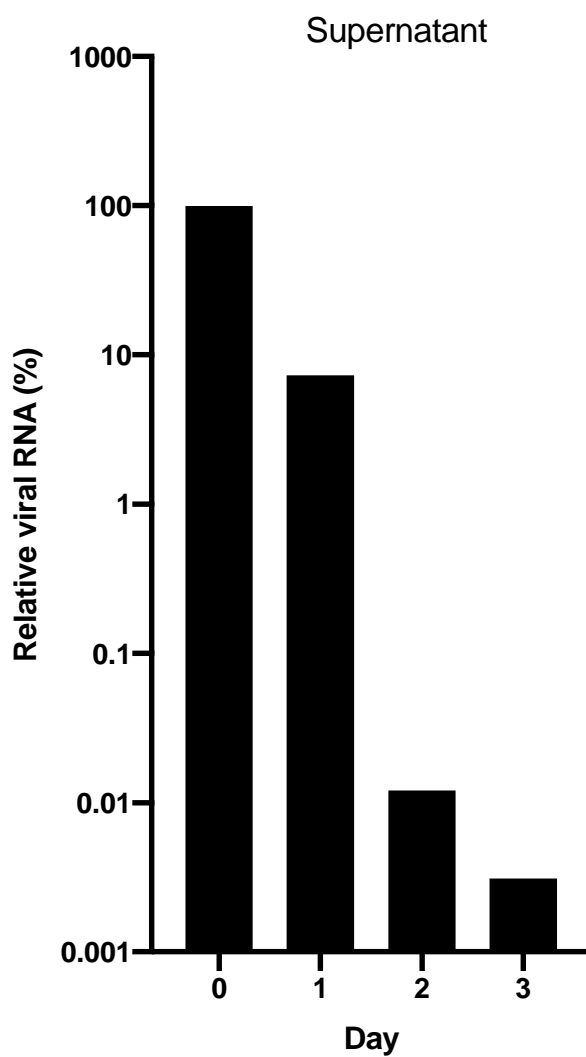
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237 **Figure 1: Ivermectin is a potent inhibitor of the SARS-CoV-2 clinical isolate**
238 **Australia/VIC01/2020.** Vero/hSLAM cells were in infected with SARS-CoV-2 clinical
239 isolate Australia/VIC01/2020 (MOI = 0.1) for 2 h prior to addition of 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 ± 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. IMPα/β1 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 Impα/β1 heterodimer thereby
252 preventing Impα/β1 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,
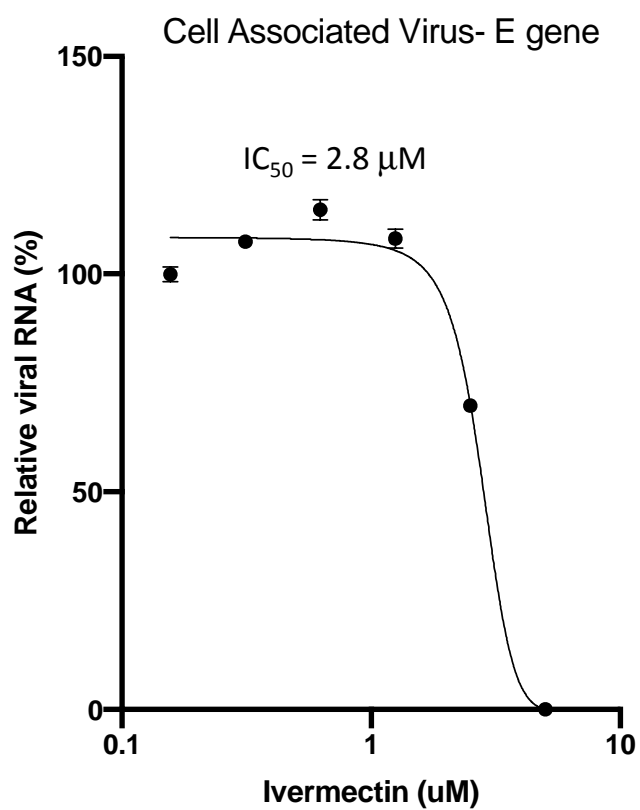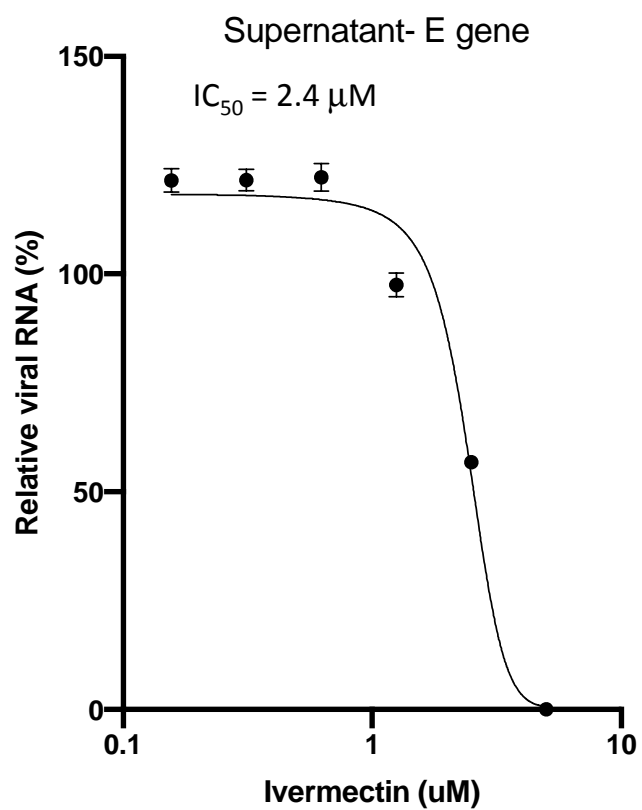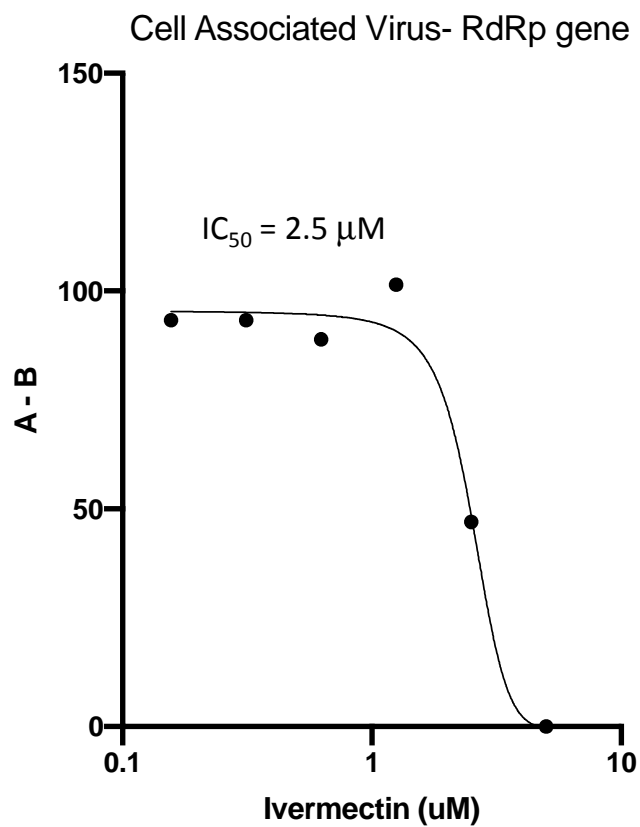254 leading to a normal, more efficient antiviral response.

255

A Cell Associated Virus

B Supernatant

C

Cell Associated Virus- E gene

IC_{50} = 2.8 μM

Relative viral RNA (%)

Ivermectin (uM)

D

Supernatant- E gene

IC_{50} = 2.4 μM

Relative viral RNA (%)

Ivermectin (uM)

E

Cell Associated Virus- RdRp gene

IC_{50} = 2.5 μM

A - B

Ivermectin (uM)

F

Supernatant- RdRp gene

IC_{50} = 2.2 μM

Relative viral RNA (%)

Ivermectin (uM)

G

CYTOPLASM

NUCLEUS

Imp β1

Imp α

Imp β1

Imp α

Coronavirus protein

NPC

Coronavirus protein

ENHANCED INFECTION/REDUCED ANTIVIRAL RESPONSE

Imp β1

Imp α

ivermectin

Coronavirus protein
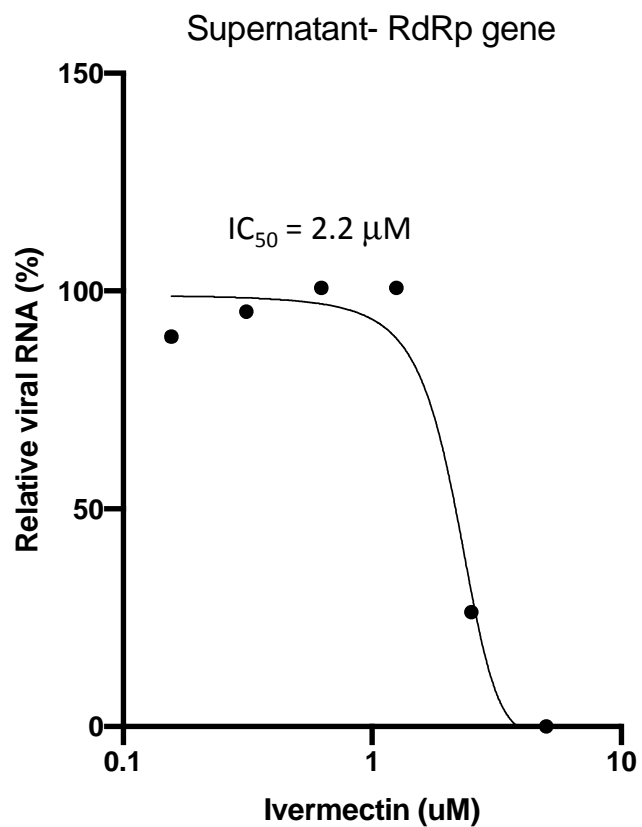
NPC

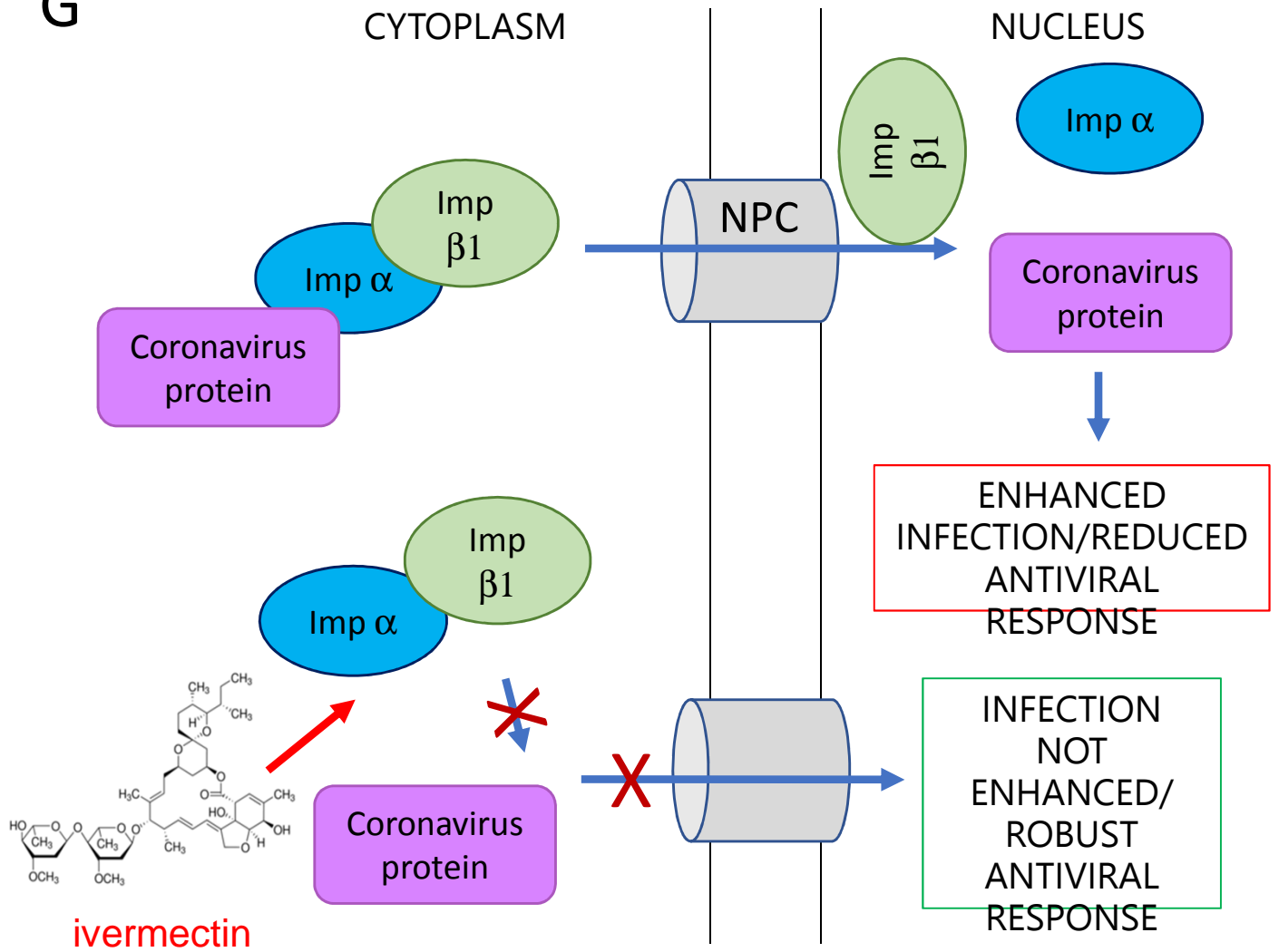INFECTION NOT ENHANCED/ ROBUST ANTIVIRAL RESPONSE

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.