1 *Short Communication*

2 **Determining the antiviral activity of two polyene macrolide antibiotics following treatment in Kidney**
3 **Cells infected with SARS-CoV-2**

4

5 Kishor M. Wasan [1,3*] and Chris Galliano [2]

6 [1] Department of Urologic Sciences, Faculty of Medicine, The University of British Columbia
7   Kishor.Wasan@ubc.ca
8 [2] Skymount Medical Inc.; chris@skymountmed.com
9 [3] Amphotericin B Technologies Inc., A subsidiary of Satellos biosciences Inc.
10
11 * Correspondence: Kishor.Wasan@ubc.ca; 306-514-1209

12 **Abstract**:

13 **Background**: There has been much speculation that polyene macrolide antibiotics, such as am-
14 photericin B (AmB) and Nystatin (NYS) may have antiviral activity against several viruses in-
15 cluding SARS-CoV-2. **Objective**: The objective of this short communication was to determine the
16 antiviral activity of two polyene macrolides, AmB and NYS, following treatment in kidney cells
17 infected with SARS-CoV-2. **Methods**: A serial dilution of AmB, NYS, and irbesartan (a drug known
18 to bind to the ACE-2 receptor as a positive control) were then added (n=4 at each concentration) to
19 the infected Vero'76 kidney cells in 100 μL media. Cells were also examined for contamination at 24
20 hours, and for cytopathic effect (CPE) and cytotoxicity (if noticeable) under a microscope at 48
21 hours. In a second study, AmB and Remdesivir were incubated in kidney cells infected with the
22 virus and inhibition of the virus was determined by an immunoassay. **Results and Conclusions**:
23 Amphotericin B (AmB) showed a significant reduction in the TCID50 titer, with the 50% effective
24 concentration (EC50) of 1.24 μM, which was 2.5 times lower than the cytotoxicity concentra-
25 tion. NYS and Irbesartan both exhibited substantially less active and would not be considered a
26 suitable choice for further investigations. In addition, when measuring viral inhibition by immu-
27 noassay, AmB was significantly more potent than remdesivir (EC50 31.8 nM vs. 1.15 μM). Taken
28 together, these preliminary findings suggest that AmB may have significant activity against
29 SARS-CoV-2. However, further cell and animal studies are warranted.

Keywords: Amphotericin B, Nystatin, Irbesartan, Antiviral Activity, SARS-CoV-2, Vero kidney cells

30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46

1. Introduction

The emergence of COVID-19 has affected every population in the world causing millions of infections. To date, there are very few therapeutics to treat patients with COVID-19 that exhibit mild or moderate symptoms but not require hospitalization. The development of an effective, safe, and proven vaccine is clearly the key step in controlling the pandemic, however, there is still a need for effective therapeutics to treat those in-fected with the virus. Amphotericin B (AmB) is a polyene macrolide antibiotic used for the treatment of systemic fungal and parasitic infections [1-10]. There has been much speculation that AmB may have antiviral activity against several viruses including the ones responsible for COVID-19 [11-15]. Recent investigations have suggested that AmB may interact with the S-protein of the SARS-CoV-2 virus, thus blocking its interaction with the ACE-2 receptor found on epithelial and endothelial cells [11-15]. Thus, the hy-pothesis of this study was that AmB will function as an antiviral not only by binding to and preventing SARS-CoV-2 virus replication, but also by blocking viral uptake into

47 mammalian cells. Our laboratory tested this hypothesis by determining the antiviral ac-
48 tivity of AmB against SARS-CoV-2 within kidney cells.
49
50

2. Materials and Methods

52 Vero'76 (ATCC CRL-1587) kidney cells were seeded and grown overnight at 37□ in
53 a 5% CO_2 environment to approximately 90% confluence in 96-well plates. The culture
54 medium used was DMEM supplemented with 10% FBS/PEN-STREP. The virus
55 SARS-CoV-2/Canada/ON/VIDO-01/2020/Vero'76/p.2 (Seq. available at GISAID –
56 EPI_ISL_413015) was diluted in DMEM supplemented with 2% FBS/PEN-STREP to ob-
57 tain a multiplicity of infection (MOI) of 0.1 (approximately 2000 $TCID_{50}$/well). The cul-
58 ture medium was removed from the cells and 50 µl of virus inoculum was added to each
59 well. The plates were incubated for 1 hour at 37□ in a 5% CO_2 environment, then the
60 medium was removed. A serial dilution of AmB, NYS, and Irbesartan (a drug known to
61 bind to the ACE-2 receptor as a positive control) were then added (n=4 at each concen-
62 tration; data presented as mean +/- SD; SD are very small of the size of the graph) to the
63 infected cells in 100 µL media. Medium alone was used as a control for virus replication,
64 and untreated, uninfected control wells were also established. A separate plate was used
65 to assess the cell toxicity of the test compounds by incubating uninfected cells with the
66 serially diluted test compound solution. After 48 hours of incubation, cytotoxicity was
67 measured by MTS assay (CellTiter 96 Aqueous One (Promega. Spectroscopic absorbance
68 at 490 nM following incubation is directly related to cytotoxicity. The corrected absorb-
69 ance at 490 nM versus the concentration of AmB, NYS, and irbesartan (n=4 at each con-
70 centration) was plotted to determine CC50, the concentration at which 50% of the cells
71 were dead. Cells were also examined for contamination at 24 hours, and for cytopathic
72 effect (CPE) and cytotoxicity (if noticeable) under a microscope at 48 hours.   At 48
73 hours, 100 µl supernatant from each well was harvested. Viral titration by the $TCID_{50}$
74 assay of the supernatant was carried out by a serial 10-fold dilution. These dilutions
75 were subsequently used to infect another set of plated cells, in quadruplicates as de-
76 scribed for the initial infection. Cells were observed for CPE at 1, 3, and 5 days after in-
77 fection.

78 In a second set of studies, efficacies were tested in parallel in African green monkey
79 kidney (Vero E6) cells. Each test compound was tested individually and each of the
80 concentrations was evaluated in triplicate for efficacy. Vero E6 cells were cultured in 96
81 plates prior to the day of the assay. Cells were greater than 90% confluency at the start of
82 the study. Each of the test article concentrations was evaluated in triplicate.   Test article
83 concentration were tested in two different conditions: 1) Pre-treatment for 24 ± 4 hours
84 prior to virus inoculation followed by treatment immediately after removal of the virus
85 inoculum or 2) treatment only with the test article added immediately following re-
86 moval of the virus inoculum. These two different conditions were tested to ascertain the
87 effect of the drugs on the entry, fusion, replication, and transmission of the virus.

88 Remdesivir was added immediately following removal of the virus inoculum. For pre-
89 treatment and treatment, wells were overlaid with 0.2 mL DMEM2 (Dulbecco's Modi-
90 fied Eagle Media (DMEM) with 2% Fetal Bovine Serum (FBS) to test articles at concen-
91 trations as delineated in Section 9.8). Following the 24 ± 4 hour pretreatment, cells were
92 inoculated at a MOI of 0.001 TCID50/cell with SARS-CoV-2 and incubated for 60-90
93 minutes. Immediately following the 60–90-minute incubation, the virus inoculum was
94 removed, cells washed, and appropriate wells overlaid with 0.2 mL DMEM2 (DMEM
95 with 2% FBS with test or control articles) and incubated in a humidified chamber at 37°C
96 ± 2°C in 5 ± 2% CO2. At 48 ± 6 hours post inoculation, cells were fixed and evaluated for

97 the presence of virus by immunostaining assay justification: The immunostaining assay
98 utilized a modified incubation time to 48 hours. A 24 ± 4-hour pre-treatment of the cells
99 was included for selected test articles. After 48 ± 6 hours, cells are fixed with parafor-
100 maldehyde and stained by anti-SARS-2 nucleoprotein monoclonal antibody (Sino Bio-
101 logical) followed by peroxidase-conjugated goat anti-mouse IgG (SeraCare). Wells are
102 developed using TMB Substrate Solution and the reaction stopped by acidification. The
103 ELISA plate were read at 450 nm on a spectrophotometer by ELISA plate reader.

104 For each well, the inhibition of virus is calculated as the percentage of reduction of the
105 absorbance value in respect to the virus control by the following formula: percent inhibi-
106 tion = 100 - [(A450 of article dilution - A450 of cell control)/(A450 of virus control - A450
107 of cell control)] x 100. The EC50 is defined as the reciprocal dilution that caused a 50%
108 reduction of the absorbance value of the virus control (50% A450 reduction).

110 **3. Results**

111 Amphotericin B (AmB) showed a significant reduction in the TCID50 titer, with the
112 50% effective concentration (EC50) of 1.24 μM, which was 2.5 times lower than the cyto-
113 toxicity concentration (**Table 1**). Nystatin (NYS), a compound similar in chemical struc-
114 ture to Amphotericin B and irbesartan both exhibited substantially less active and would
115 not be considered a suitable choice for further investigations. Figures 1 through 3 dem-
116 onstrate the difference in concentration response of NYS and irbesartan compared to
117 AmB, respectively, where AmB exhibits a 32-fold lower concentration required to
118 achieve EC50 at a 15-fold lower CC50 compared to nystatin. The selectivity index (SI)
119 was calculated as the ratio of CC50 to EC50. The SI for both compounds was >1, which
120 indicates that the compounds are more effective than they are toxic to the cells (**Table 1**).

121 **Table 1-** **Vero-76 Kidney Cells Infected Cells (Viral**
122 **Strain: (SARS-CoV-2/Canada/ON/VIDO-01/2020/Vero'76/p.2)* treated with Amphotericin B, Nys-**
123 **tatin and Irbesartan.**

Drug	EC$_{50}$	SI
Amphotericin B	1.24 μM **	3.1
Nystatin	36.6 μM	1.41
Irbesartan	62.5 μM	5.55

126 *D614G variant that has been circulating across the continent since early 2020 and there have been no

127 appreciable differences in transmission or virulence across cities in North America that would

128 suggest any mutational changes in the virus resulting in a new

129 variant.   **Activity based on concentrations used to treat systemic fungal infections (1000-2000 nM).

131 **Table 2,** **Efficacy in African green monkey kidney (Vero E6) cells infected with Virus (Viral Strain :2019**
132 **Novel Coronavirus, Isolate USA-WA1/2020 (SARS-CoV-2)Code**

Drug	EC$_{50}$	EC$_{100}$	comment
Amphotericin B	31.8 nM**	Achieved	

Remdesevir	1.15um	Achieved	Positive control

133 **Activity based on concentrations used to treat systemic fungal infections (1000-2000 nM).

134

135   The above results of **Table 2** confirm the results obtained in the first testing series and confirm the

136 versatility of AmB as a replication blocker of COVID-19 as the tests were carried out on different

137 strains of COVID-19.   The above results also indicate that in this test, AmB was clearly superior to

138 remdesevir in terms of EC50.

139

140 **Figure 1**. Virus titer and cell viability following increasing concentrations of nystatin have been incubated with Vero

141 kidney cells for 48 hours that have been infected with the SARS-CoV-2 virus. Data is presented as mean +/- standard

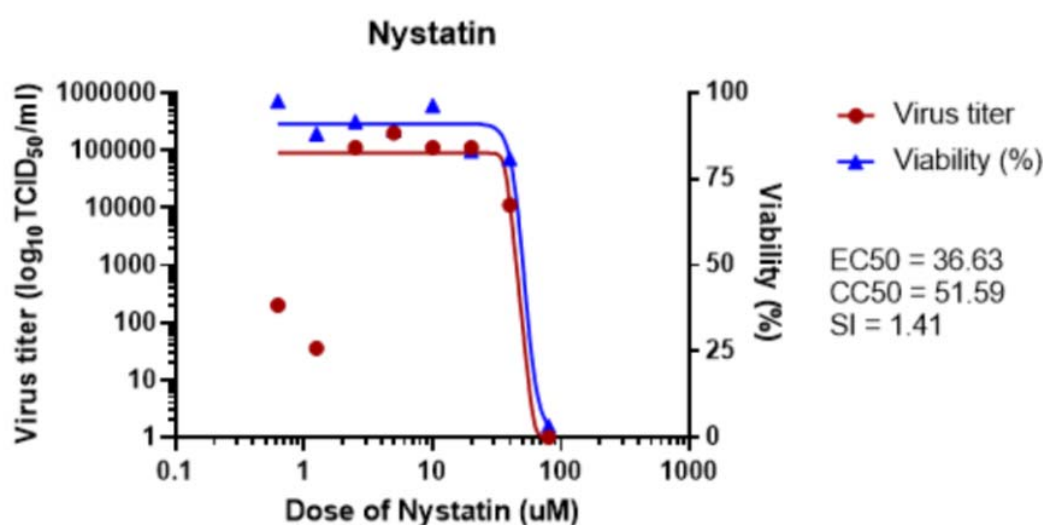142 deviation (n=4 for each concentration tested).

143

144 **Figure 2.** Virus titer and cell viability following increasing concentrations of irbesartan have been incubated with

145 Vero kidney cells for 48 hours that have been infected with the SARS-CoV-2 virus. Data is presented as mean +/-

146 standard deviation (n=4 for each concentration tested).
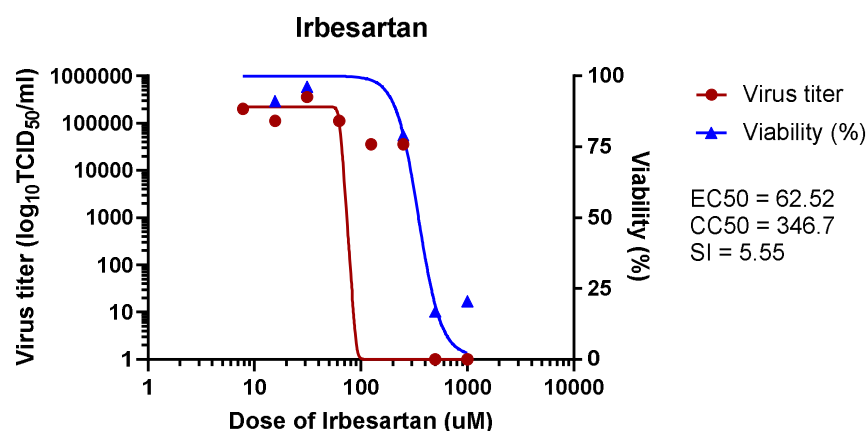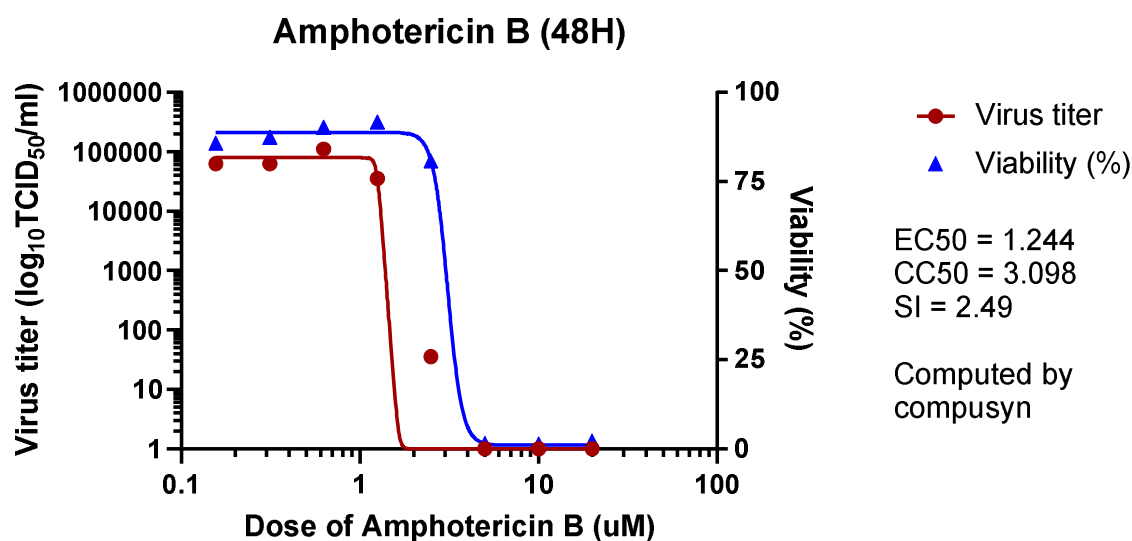
147

148

149

Figure 3. Virus titer and cell viability following increasing concentrations of Amphotericin B have been incubated with Vero kidney cells for 48 hours that have been infected with the SARS-CoV-2 virus. Data is presented as mean +/- standard deviation (n=4 for each concentration tested).

153

154

155

4. Discussion and Conclusions

Taken together, these preliminary findings suggest that AmB may have significant activity against SARS CoV2 and may be efficacious in patients suffering from COVID-19 where better treatment is still needed. Further cell and animal studies are required to

confirm these important preliminary findings and could lead to the development and implementation of AmB in the treatment of COVID 19.

References

1. Wasan K. Development of an Oral Amphotericin B Formulation as an Alternative Approach to Parenteral Amphotericin B Administration in the Treatment of Blood-Borne Fungal Infections [published online ahead of print, 2020 Mar 11]. *Curr Pharm Des.* 2020;10.2174/1381612826666200311130812. doi:10.2174/1381612826666200311130812

2. Cuddihy G, Wasan EK, Di Y, Wasan KM. The Development of Oral Amphotericin B to Treat Systemic Fungal and Parasitic Infections: Has the Myth Been Finally Realized? *Pharmaceutics.* 2019;11(3):99.

3. Kleinberg M. (2006). What is the current and future status of conventional amphotericin B. Int J Antimicrob Agents. Jun;27 Suppl 1:12-6.

4. Sachs-Barrable K, Wasan KM. (2008). Enhancing drug absorption using lipids: A case study presenting the development and pharmacological evaluation of a novel lipid-based oral amphotericin B formulation for the treatment of systemic fungal infections. Advanced Drug Delivery Reviews. 60: 692–701.

5. Ibrahim F, Wasan KM. (2012). Assessment of novel oral lipid-based formulations of amphotericin B using an in vitro lipolysis model. Eur J Pharm Sci. Aug 15;46(5):323-8.

6. Gershkovich P, Wasan KM. (2009). Pharmacokinetics and biodistribution of amphotericin B in rats following oral administration in a novel lipid-based formulation. Journal of Antimicrobial Agents and Chemotherapy. 64(1): 101-108

7. Wasan EK, Gershkovich P, Zhao J, Wasan KM. A novel tropically stable oral amphotericin B formulation (iCo-010) exhibits efficacy against visceral Leishmaniasis in a murine model. *PLoS Negl Trop Dis.* 2010;4(12):e913.

8. Wasan KM, Wasan EK, Gershkovich P, Wasan KM. Highly effective oral amphotericin B formulation against murine visceral leishmaniasis. *J Infect Dis.* 2009;200(3):357-360.

9. Jensen G.M. The care and feeding of a commercial liposomal product: Liposomal amphotericin B (AmBisome((R))) J. Liposome Res. 2017;27:173–179. doi: 10.1080/08982104.2017.1380664. - DOI - PubMed

10. Ling Tan J.S., Roberts C.J., Billa N. Mucoadhesive chitosan-coated nanostructured lipid carriers for oral delivery of amphotericin B. Pharm. Dev. Technol. 2018;26:1–9. doi: 10.1080/10837450.2018.1515225. - DOI - PubMed

11. Al-Khikani FHO. Amphotericin B as antiviral drug: Possible efficacy against COVID-19. Ann Thorac Med. 2020 Jul-Sep;15(3):118-124. doi: 10.4103/atm.ATM_147_20. Epub 2020 Jun 18. PMID: 32831932; PMCID: PMC7423209.

203 12. Kalhor H, Sadeghi S, Abolhasani H, Kalhor R, Rahimi H. Repurposing of the approved small molecule drugs
204   in order to inhibit SARS-CoV-2 S protein and human ACE2 interaction through virtual screening approaches. J
205   Biomol Struct Dyn. 2020 Sep 24:1-16. doi: 10.1080/07391102.2020.1824816. Epub ahead of print. PMID:
206   32969333; PMCID: PMC7576931.
207 13. Qiao Z, Zhang H, Ji HF, Chen Q. Computational View toward the Inhibition of SARS-CoV-2 Spike Glycopro-
208   tein and the 3CL Protease. Computation (Basel). 2020 Jun;8(2):53. doi: 10.3390/computation8020053. Epub 2020
209   May 31. PMID: 32661494; PMCID: PMC7357730.
210 14. Zhao X, Zheng S, Chen D, Zheng M, Li X, Li G, Lin H, Chang J, Zeng H, Guo JT. LY6E Restricts Entry of Hu-
211   man Coronaviruses, Including Currently Pandemic SARS-CoV-2. J Virol. 2020 Aug 31;94(18):e00562-20. doi:
212   10.1128/JVI.00562-20. PMID: 32641482; PMCID: PMC7459569.
213 15. Zheng M, Zhao X, Zheng S, Chen D, Du P, Li X, Jiang D, Guo JT, Zeng H, Lin H. Bat SARS-Like WIV1 coro-
214   navirus uses the ACE2 of multiple animal species as receptor and evades IFITM3 restriction *via* TMPRSS2 ac-
215   tivation of membrane fusion. Emerg Microbes Infect. 2020 Dec;9(1):1567-1579. doi:
216   10.1080/22221751.2020.1787797. PMID: 32602823; PMCID: PMC7473123.

217