1 **Arbidol (Umifenovir): A Broad-spectrum Antiviral Drug that Inhibits Medically Important**
2 **Arthropod-borne Flaviviruses**

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20 **Abstract:** Arthropod-borne flaviviruses represent human pathogens of global medical
21 importance, against which no effective small molecule-based antiviral therapy is currently
22 available. Arbidol (umifenovir) is a broad spectrum antiviral compound approved in Russia
23 and China for prophylaxis and treatment of influenza. This compound showed activity
24 against numerous DNA and RNA viruses. Its mode of action is based predominantly on the
25 impairment of critical steps of virus-cell interaction. Here we demonstrate that arbidol
26 possesses a micromolar inhibition activity (EC$_{50}$ values ranging from 10.57 ± 0.74 to 19.16 ±
27 0.29 μM) in Vero cells infected with Zika virus, West Nile virus, and tick-borne encephalitis
28 virus, three medically important representatives of arthropod-borne flaviviruses.
29 Interestingly, no antiviral effect of arbidol is observed in porcine stable kidney cells (PS),
30 human neuroblastoma cells (UKF-NB-6), human hepatoma cells (Huh-7 cells) indicating that
31 the antiviral effect of arbidol is strongly cell-type dependent. Arbidol presents a significant
32 increasing in cytotoxicity profiles when tested in various cell lines in the order: Huh-7 <
33 HBCA < PS < UKF-NB-6 < Vero with CC$_{50}$ values ranging from 18.69 ± 0.1 to 89.72 ± 0.19 μM.
34 Antiviral activity and acceptable cytotoxicity profiles suggest that arbidol could be a
35 promising candidate for further investigation as a potential therapeutic agent in treating
36 flaviviral infections.
37 **Keywords:** flavivirus; arbidol; umifenovir; antiviral activity; cytotoxicity; cell-type dependent
38 antiviral effect
39

1. Introduction

40

41   Arthropod-borne flaviviruses (genus *Flavivirus*, family *Flaviviridae*) include human
42   pathogens of global medical importance such as dengue virus (DENV), Yellow fever virus
43   (YFV), West Nile virus (WNV), Japanese encephalitis virus (JEV), Zika virus (ZIKV), Kyasanur
44   Forest disease virus (KFDV), Omsk haemorrhagic fever virus (OHFV) and tick-borne
45   encephalitis virus (TBEV). These viruses are causative agents of many serious diseases with a
46   broad-spectrum of clinical symtoms ranging from asymptomatic or mild flu-like infections
47   through neurological diseases (WNV, TBEV) to viscerotropic (DENV), haemorrhagic (KFDV,
48   OHFV) or terratogenic manifestations (ZIKV) [1;2]. Up to 200 million new cases of infections
49   caused by arthropod-borne flaviviruses are reported annually [1]. These viruses can be
50   widespread unexpectedly, as exemplified by the epidemiological outbreaks of WNV infection
51   across North America, Mexico, South America, and the Caribbean during 1999 – 2002 [3;4],
52   or ZIKV infection in Oceania and Latin America during 2014 – 2016 [5]. At present, there is no
53   effective antiviral therapy directed against these viruses, and therefore, search for small
54   molecule-based inhibitors represents an unmet medical need.

55   Arbidol also known as umifenovir, is a broad spectrum antiviral compound developed
56   at the Russian Research Chemical and Pharmaceutical Institute about 25 years ago [6] and
57   licensed in Russia and China for prophylaxis and treatment of human influenza A and B
58   infections and post-influenza complications [7]. Arbidol was later shown to be active against
59   numerous DNA/RNA and enveloped/non-enveloped viruses [8]. The predominant mode of
60   action for arbidol is based on its intercalation into membrane lipids and on the subsequent
61   inhibition of membrane fusion between the virus and the plasma membrane and also
62   between the virus and the endocytic vesicle membrane [9]. In influenza virus, arbidol was
63   observed to interact with virus hemagglutinin (HA); this interaction results in an increased
64   HA stability and prevents pH-induced HA transition to its fusogenic state [10]. In the case of
65   hepatitis C virus (HCV) arbidol interacts with HCV envelope protein to cause various degree
66   of fusion inhibition [11;12]. In addition to these common critical steps in virus-cell
67   interaction, arbidol was also described to show some immunomodulatory activities, such as
68   interferon induction effect and macrophage activation [13]. Due to its broad-spectrum
69   antiviral activity arbidol represents a promising candidate for treatment of viral infections in
70   humans.

71   Using standardised *in vitro* assay systems, this study compares the cytotoxicity and

72   antiviral activity of arbidol against three representative flaviviruses; ZIKV and WNV as

73   emerging mosquito-borne pathogens; TBEV as an important tick-borne pathogen. As many

74   antiviral compounds are extensively inactivated/metabolised intracellularly [14;15], different

75   cell lines were utilised for assessment of antiviral and cytotoxic effects of arbidol.

76

77   **2. Material and methods**

78   Arbidol   (ethyl-6-bromo-4-[(dimethylamino)methyl]-5-hydroxy-1-methyl-2-

79   [(phenylthio)methyl]-indole-3-carboxylate hydrochloride monohydrate) (Figure 1A) was

80   obtained from Sigma-Aldrich, solubilized in 100% DMSO to yield 10 mM stock solution. The

81   following viral strains/isolates were used in this study: ZIKV (MR766, a representative of the

82   African ZIKV lineage; Paraiba_01, a member of the Asian ZIKV lineage), WNV (strains Eg101

83   and 13-104), and TBEV (strain Hypr, a typical representative of the West European TBEV

84   subtype). As ZIKV, WNV and TBEV are neurotropic viruses, cell lines of both neuronal as well

85   as extraneural origin were selected for antiviral screens, virus multiplication and plaque

86   assays. Porcine kidney stable (PS) cells [16] were cultured in Leibovitz (L-15) medium, human

87   brain cortical astrocytes (HBCA) (ScienCell, Carlsbad, CA) were cultivated in Astrocyte

88   medium (Thermo Fisher Scientific), human neuroblastoma UKF-NB-4 cells [17] were cultured

89   in Iscove's modified Dulbecco's medium (IMDM), Vero cells (ATCC CCL-81, African Green

90   Monkey, adult kidney, epithelial) and human hepatocellular carcinoma cells (Huh-7) were

91   grown in Dulbecco's modified Eagle's medium (DMEM). The media were supplemented with

92   3% (L-15), 6% (Astrocyte medium), or 10% (IMDM and DMEM) newborn calf serum and a 1%

93   mixture of antibiotics and antimycotics and 1% glutamine (Sigma-Aldrich, Prague, Czech

94   Republic).

95   The compound cytotoxicity was determined in terms of cell viability using the Cell

96   Counting Kit-8 (Dojindo Molecular Technologies, Munich, Germany) according to the

97   manufacturer's instructions and expressed as the 50% cytotoxic concentration (CC_{50}), which

98   represents the concentration of compound that reduced cell viability by 50%. A viral titre

99   reduction assay was performed to determine the flavivirus sensitivity to arbidol in cell

100   culture. Host cells were seeded in 96-well plates (approximately 2×10^4 cells per well), and

101   incubated for 24 h at 37 °C to form a confluent monolayer. The medium was then aspirated

102   from the wells and replaced with 200 μl of fresh medium containing 0 – 12.5 μM (for Huh-7),

103   0 - 25 µM (for HBCA and PS), 0 – 30 µM (for UKF-NB-6), or 0—50 µM (for Vero) of arbidol

104   and incubated for 24 hours (the concentration ranges were based on different cytotoxicity

105   effects of arbidol for individual cell lines, as described below). The medium was then

106   removed from wells and replaced with fresh medium containing arbidol and appropriate

107   virus strain at a multiplicity of infection (MOI) of 0.1. After 2 h incubation, the medium was

108   replaced with fresh medium containing arbidol and incubated for 48 h at 37 °C. Then, the

109   supernatant medium was collected and viral titres were determined by plaque assays,

110   expressed as PFU/mL [18] and used for construction of dose-response and inhibition curves

111   and for estimation the 50% effective concentration (EC_{50}). A cell-based flavivirus

112   immunostaining assay was performed to measure the arbidol-induced inhibition of viral

113   surface antigen (E protein) expression, as previously described [19].

114

115   ### 3.   Results and discussion

116   We initially determined the cytotoxicity of arbidol for Huh-7, Vero, PS, UKF-NB-6, and

117   HBCA cells. As apparent from Figure 1B, arbidol presented significant differences in

118   cytotoxicity profiles when tested in various cell lines. Arbidol showed the lowest cytotoxicity

119   in Vero cells (CC_{50} = 89.72 ± 0.19 µM), about 5 times lower than in Huh-7 cells (CC_{50} = 18.69 ±

120   0.1 µM) in which the highest cytotoxic effect was observed. The other cell types PS, UKF-NB-

121   6, and HBCA had intermediate CC_{50} values ranging from 24.78 ± 0.01 to 46.99 ± 0.1 µM

122   (Table 1). The variable cytotoxicity of arbidol may be related to its broad-spectrum activity

123   impairing crucial cellular metabolic pathways or critical steps in virus-cell interactions

124   [11;20]. This is in contrast to antiviral drugs targeting preferentially viral proteins, such as

125   nucleoside inhibitors of viral polymerases, for which the CC_{50} values usually do not exceed

126   100 µM [21].

127   The antiviral effect of arbidol against two ZIKV strains (MR766 and Paraiba_01) was

128   evident in both Vero and HBCA cells 48 h after infection. Whereas in HBCA the highest

129   arbidol concentration tested (25 µM) caused a ZIKV titre reduction of about 10^4-fold

130   compared to mock-treated cells, in Vero cells we observed a complete inhibition of viral

131   replication at 50 µM. No inhibition of ZIKV replication was observed in UKF-NB-6, PS, and

132   Huh-7 cells, indicating that the antiviral effect of arbidol is strongly cell type-dependent

133   (Figure 1C). This phenomenon has been previously described for many antiviral compounds

134   and is based on different in expression levels of intracellular enzymes/proteins involved in

135   compound metabolism and transport [21]. This results in different EC_{50} values for the same

136   inhibitor when assayed on different cell lines [22;23].

137   Based on these results, we further evaluated the arbidol inhibitory potential on the

138   most efficient Vero cells. From the ZIKV inhibition curves, the EC_{50} values were determined

139   to be 12.09 ± 0.77 and 10.57 ± 0.74 µM for MR766 and Paraiba_01 strains, respectively

140   (Figure 1D,E, Table 2). The antiviral effect of arbidol was further confirmed by

141   immunofluorescence staining; this method revealed the dose-dependent inhibition effect of

142   arbidol on the expression of the ZIKV surface E antigen in Vero cells (Figure 2). The observed

143   anti-ZIKV properties are comparable with those for previously reported small-molecule, such

144   as nucleoside analogues, which exerted anti-ZIKV activities in various cell-based assay

145   systems around the micromolar range (0.2 to 22 µM) [24-27].

146   Arbidol also showed a significant in vitro antiviral efficacy when tested against two

147   strains of WNV (Eg101 and 13-104). Similarly to ZIKV, the anti-WNV effect of arbidol was

148   most obvious when the compound at concentrations 25 and 50 µM was assayed in WNV-

149   infected HBCA and Vero cells after 48h incubation, respectively, and there was no or

150   negligible antiviral effect seen in virus-infected UKF-NB-6, PS, and Huh-7 cells (Figure 1C).

151   Although, these concentrations of arbidol resulted in the reduction WNV titres 10^3-fold

152   compared to non-treated cells, they did not lead to a complete inhibition of virus replication

153   in cell culture (Figure 1C). The incomplete inhibition of viral growth was reflected on the EC_{50}

154   values (18.78 ± 0.21 and 19.16 ± 0.29 µM for Eg101 and 13-104, respectively), which were

155   slightly higher (about two-times) compared with those for ZIKV (Figure 1D,E, Table 2). The

156   observed arbidol-mediated decreasing of WNV titre strongly correlated with dose-

157   dependent inhibition of viral surface E antigen expression in the compound-treated Vero cell

158   culture (Figure 2).

159   Finally, HBCA and Vero cells were also proven suitable to assess the inhibitory effect

160   of arbidol on TBEV (strain Hypr) (Figure 1C). Arbidol inhibited TBEV in dose-dependent

161   manners with an EC_{50} value of 18.67 ± 0.15 µM (Figure 1D,E, Table 2). Similarly to WNV, the

162   highest applied concentration (25 µM for HBCA and 50 µM for Vero cells) resulted in an

163   incomplete inhibition of TBEV replication in cell culture, although the viral titre was reduced

164   by 10^3-fold compared with non-treated cells (Figure 1C, Figure 2).

165   Our data are in agreement with numerous reports on in vitro inhibitory activities of

166   arbidol against other viruses of medical interest. It inhibits replication of various subtypes of

167   human influenza A and B viruses with EC_{50} values ranging from 3 to 9 μg/mL [28]. Arbidol

168   also shows in vitro antiviral effect on Chikungunya virus replication in Vero cells or primary

169   human fibrolasts with EC_{50} values < 10 μg/mL [29] and on Crimean-Congo haemorrhagic

170   fever virus with EC_{50} of 2.8 μg/mL [30]. Pre-treatment of human hepatocellular carcinoma

171   cells Huh 7.5.1 with 15 μM of arbidol for 24h to 48h inhibited HCV replication by up to 10^3-

172   fold [11] Indeed, arbidol exhibited a broad range antiviral effect against respiratory syncytial

173   virus, hepatitis B virus, adenovirus, parainfluezna virus, avian coronavirus, coxackie B3 virus

174   and many other viruses, indicating [11]. Beside in vitro studies, arbidol exerted also

175   substantial antiviral effect in various animal models and was highly effective in clinical trials

176   for prevention and treatment of influenza [31]. Recently, arbidol was reported to induce no

177   embryo toxicity in pregnant female rats, nor to alter the reproductive function of animals,

178   over a 20 day oral administration period of 500 mg/kg doses [32]. Based on these results we

179   can speculate that arbidol might be a promising candidate for effective treatment of viral

180   infections during pregnancy in humans, which could be relevant particularly for pregnant

181   ZIKV patients.

182

183   **4. Conclusion**

184   In conclusion, we demonstrated that arbidol show a substantial in vitro antiviral

185   activity against ZIKV, WNV and TBEV with EC_{50} values ranging from 10.57 ± 0.74 to 19.16 ±

186   0.29 μM. The observed antiviral effect is strongly cell-type dependent being obvious only in

187   HBCA and Vero cells, which could be ascribed to differences in compound up-take or

188   metabolic processing of arbidol by individual cell lines tested. Arbidol exerts also significant

189   differences in cytotoxicity profiles when tested in various cell lines; this compound is the

190   most toxic for human hepatoma cells Huh-7, whereas the lowest cytotoxicity was observed

191   for Vero cells. Promising data obtained by our in vitro antiviral screens open the possibility

192   for future testing of antiviral efficacy of arbidol in animal models.

193

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199

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Authors' contributions

207 DR, LE, NT and SK designed and conceived this study. JH, MS and MF performed the

208 experiments. IR and ZH contributed new reagents/analytic tools. LE, DR and NT wrote the

209 manuscript. All authors read and approved the manuscript.

210

Competing interests

212 The authors declare that they have no competing interests.

213

Consent for publication

215 Not applicable.
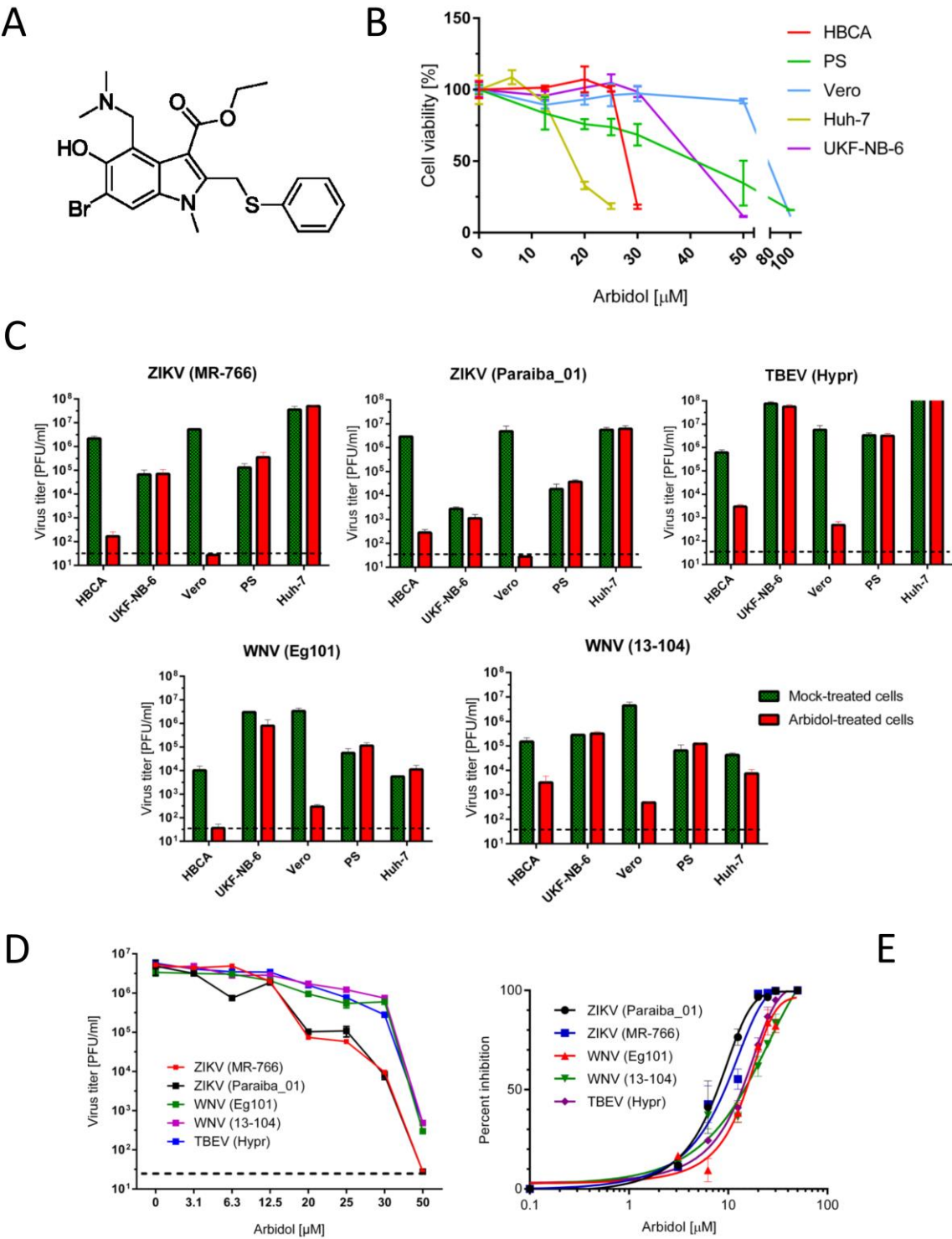
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Figures

218

Figure 1

220   (A) Structure of arbidol. (B) Cytotoxicity of arbidol for Huh-7, PS, UKF-NB-6, HBCA, and Vero

221   cells within the compound concentration range 0 -100 uM at 48h post infection. (C) Antiviral

222   effect of arbidol for ZIKV, WNV and TBEV on different cell lines. As arbidol caused different

223   cytotoxicity for individual cell types, the indicated cell lines were treated with different

224   compound concentrations (12.5 µM for Huh-7, 25 µM for HBCA and PS, 30 µM for UKF-NB-6,

225   and 50 µM for Vero) and infected 24h later with the particular virus. Culture supernatants

226   were collected 48h post infection and the viral titres were determined by plaque assay. (D)

227   Dose-dependent effect of arbidol on virus titres at 48h post infection on Vero cell culture.

228   The horizontal dashed line indicates the minimum detectable threshold of 1.44 $\log_{10}$

229   PFU/mL. (E) Inhibition curves of indicated flaviviruses in the presence of a serial dilution of
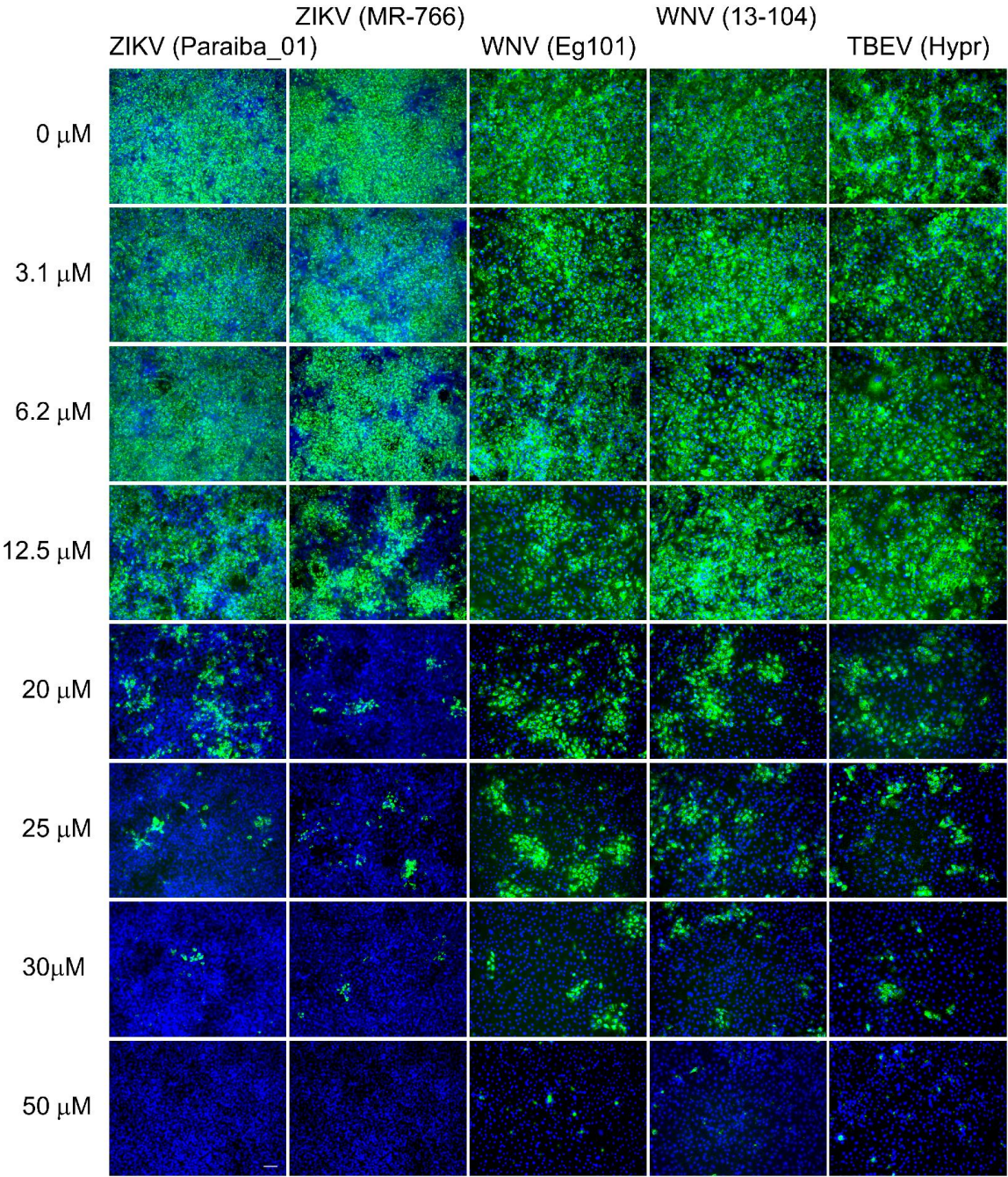
230   arbidol.

231

Figure 2

Inhibition of flaviviral surface E antigen expression by arbidol. Virus infected Vero cells were fixed on slides at 48h after infection and stained with flavivirus-specific antibody labeled with FITC (green) and counterstained with DAPI (blue). Scale bar, 50 μm.

241   **Table 1** Cytotoxicity of arbidol for various cell lines of neurone or extraneural origin
242

Cell line	CC_{50} (μM) [a]
Human brain cortical astrocytes (HBCA)	24.78 ± 0.01
Human neuroblastoma UKF-NB-4 cells	46.99 ± 0.10
Vero cells	89.72 ± 0.19
Human hepatocarcinoma cells (Huh-7)	18.69 ± 0.10
Porcine kidney stable cells (PS)	46.81 ± 1.65

243   [a] Determined from three independent experiments.
244

245   **Table 2** Anti-flaviviral activity and cytotoxicity characteristics of arbidol in Vero cells
246

Virus	Strain	EC_{50} (μM)[a,b]	CC_{50} (μM)[a,c]	SI (CC_{50}/EC_{50})
ZIKV	MR-766	12.09 ± 0.77		7.42
	Paraiba_01	10.57 ± 0.74		8.49
WNV	Eg101	18.78 ± 0.21	89.72 ± 0.19	4.78
	13-104	19.16 ± 0.29		4.68
TBEV	Hypr	18.67 ± 0.15		4.81

247   [a] Determined from three independent experiments.
248   [b] Calculated as a 50% reduction of viral titers using the Reed-Muench method.
249   [c] CC_{50} value determined for Vero cells.
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