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

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Abstract: Arthropod-borne flaviviruses represent human pathogens of global medical importance, against which no effective small molecule-based antiviral therapy is currently available. Arbidol (umifenovir) is a broad spectrum antiviral compound approved in Russia and China for prophylaxis and treatment of influenza. This compound showed activity against numerous DNA and RNA viruses. Its mode of action is based predominantly on the impairment of critical steps of virus-cell interaction. Here we demonstrate that arbidol possesses a micromolar inhibition activity (EC50 values ranging from 10.57 ± 0.74 to 19.16 ± 0.29 µM) in Vero cells infected with Zika virus, West Nile virus, and tick-borne encephalitis virus, three medically important representatives of arthropod-borne flaviviruses. Interestingly, no antiviral effect of arbidol is observed in porcine stable kidney cells (PS), human neuroblastoma cells (UKF-NB-6), human hepatoma cells (Huh-7 cells) indicating that the antiviral effect of arbidol is strongly cell-type dependent. Arbidol presents a significant increasing in cytotoxicity profiles when tested in various cell lines in the order: Huh-7 < HBCA < PS < UKF-NB-6 < Vero with CC50 values ranging from 18.69 ± 0.1 to 89.72 ± 0.19 µM. Antiviral activity and acceptable cytotoxicity profiles suggest that arbidol could be a promising candidate for further investigation as a potential therapeutic agent in treating flaviviral infections.

Keywords: flavivirus; arbidol; umifenovir; antiviral activity; cytotoxicity; cell-type dependent antiviral effect
1. Introduction

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

Arbidol also known as umifenovir, is a broad spectrum antiviral compound developed at the Russian Research Chemical and Pharmaceutical Institute about 25 years ago [6] and licensed in Russia and China for prophylaxis and treatment of human influenza A and B infections and post-influenza complications [7]. Arbidol was later shown to be active against numerous DNA/RNA and enveloped/non-enveloped viruses [8]. The predominant mode of action for arbidol is based on its intercalation into membrane lipids and on the subsequent inhibition of membrane fusion between the virus and the plasma membrane and also between the virus and the endocytic vesicle membrane [9]. In influenza virus, arbidol was observed to interact with virus hemagglutinin (HA); this interaction results in an increased HA stability and prevents pH-induced HA transition to its fusogenic state [10]. In the case of hepatitis C virus (HCV) arbidol interacts with HCV envelope protein to cause various degree of fusion inhibition [11;12]. In addition to these common critical steps in virus-cell interaction, arbidol was also described to show some immunomodulatory activities, such as interferon induction effect and macrophage activation [13]. Due to its broad-spectrum antiviral activity arbidol represents a promising candidate for treatment of viral infections in humans.
Using standardised in vitro assay systems, this study compares the cytotoxicity and antiviral activity of arbidol against three representative flaviviruses; ZIKV and WNV as emerging mosquito-borne pathogens; TBEV as an important tick-borne pathogen. As many antiviral compounds are extensively inactivated/metabolised intracellularly [14;15], different cell lines were utilised for assessment of antiviral and cytotoxic effects of arbidol.

2. Material and methods

Arbidol (ethyl-6-bromo-4-[(dimethylamino)methyl]-5-hydroxy-1-methyl-2-[(phenylthio)methyl]-indole-3-carboxylate hydrochloride monohydrate) (Figure 1A) was obtained from Sigma-Aldrich, solubilized in 100% DMSO to yield 10 mM stock solution. The following viral strains/isolates were used in this study: ZIKV (MR766, a representative of the African ZIKV lineage; Paraiba_01, a member of the Asian ZIKV lineage), WNV (strains Eg101 and 13-104), and TBEV (strain Hypr, a typical representative of the West European TBEV subtype). As ZIKV, WNV and TBEV are neurotropic viruses, cell lines of both neuronal as well as extraneural origin were selected for antiviral screens, virus multiplication and plaque assays. Porcine kidney stable (PS) cells [16] were cultured in Leibovitz (L-15) medium, human brain cortical astrocytes (HBCA) (ScienCell, Carlsbad, CA) were cultivated in Astrocyte medium (Thermo Fisher Scientific), human neuroblastoma UKF-NB-4 cells [17] were cultured in Iscove's modified Dulbecco's medium (IMDM), Vero cells (ATCC CCL-81, African Green Monkey, adult kidney, epithelial) and human hepatocellular carcinoma cells (Huh-7) were grown in Dulbecco's modified Eagle's medium (DMEM). The media were supplemented with 3% (L-15), 6% (Astrocyte medium), or 10% (IMDM and DMEM) newborn calf serum and a 1% mixture of antibiotics and antimycotics and 1% glutamine (Sigma-Aldrich, Prague, Czech Republic).

The compound cytotoxicity was determined in terms of cell viability using the Cell Counting Kit-8 (Dojindo Molecular Technologies, Munich, Germany) according to the manufacturer’s instructions and expressed as the 50% cytotoxic concentration (CC₅₀), which represents the concentration of compound that reduced cell viability by 50%. A viral titre reduction assay was performed to determine the flavivirus sensitivity to arbidol in cell culture. Host cells were seeded in 96-well plates (approximately 2 × 10⁴ cells per well), and incubated for 24 h at 37 °C to form a confluent monolayer. The medium was then aspirated from the wells and replaced with 200 µl of fresh medium containing 0 – 12.5 µM (for Huh-7),...
0 - 25 µM (for HBCA and PS), 0 – 30 µM (for UKF-NB-6), or 0—50 µM (for Vero) of arbidol and incubated for 24 hours (the concentration ranges were based on different cytotoxicity effects of arbidol for individual cell lines, as described below). The medium was then removed from wells and replaced with fresh medium containing arbidol and appropriate virus strain at a multiplicity of infection (MOI) of 0.1. After 2 h incubation, the medium was replaced with fresh medium containing arbidol and incubated for 48 h at 37 °C. Then, the supernatant medium was collected and viral titres were determined by plaque assays, expressed as PFU/mL [18] and used for construction of dose-response and inhibition curves and for estimation the 50% effective concentration (EC$_{50}$). A cell-based flavivirus immunostaining assay was performed to measure the arbidol-induced inhibition of viral surface antigen (E protein) expression, as previously described [19].

3. Results and discussion

We initially determined the cytotoxicity of arbidol for Huh-7, Vero, PS, UKF-NB-6, and HBCA cells. As apparent from Figure 1B, arbidol presented significant differences in cytotoxicity profiles when tested in various cell lines. Arbidol showed the lowest cytotoxicity in Vero cells (CC$_{50}$ = 89.72 ± 0.19 µM), about 5 times lower than in Huh-7 cells (CC$_{50}$ = 18.69 ± 0.1 µM) in which the highest cytotoxic effect was observed. The other cell types PS, UKF-NB-6, and HBCA had intermediate CC$_{50}$ values ranging from 24.78 ± 0.01 to 46.99 ± 0.1 µM (Table 1). The variable cytotoxicity of arbidol may be related to its broad-spectrum activity impairing crucial cellular metabolic pathways or critical steps in virus-cell interactions [11;20]. This is in contrast to antiviral drugs targeting preferentially viral proteins, such as nucleoside inhibitors of viral polymerases, for which the CC$_{50}$ values usually do not exceed 100 µM [21].

The antiviral effect of arbidol against two ZIKV strains (MR766 and Paraiba_01) was evident in both Vero and HBCA cells 48 h after infection. Whereas in HBCA the highest arbidol concentration tested (25 µM) caused a ZIKV titre reduction of about 10$^4$-fold compared to mock-treated cells, in Vero cells we observed a complete inhibition of viral replication at 50 µM. No inhibition of ZIKV replication was observed in UKF-NB-6, PS, and Huh-7 cells, indicating that the antiviral effect of arbidol is strongly cell type-dependent (Figure 1C). This phenomenon has been previously described for many antiviral compounds and is based on different in expression levels of intracellular enzymes/proteins involved in
compound metabolism and transport [21]. This results in different EC\textsubscript{50} values for the same inhibitor when assayed on different cell lines [22;23].

Based on these results, we further evaluated the arbidol inhibitory potential on the most efficient Vero cells. From the ZIKV inhibition curves, the EC\textsubscript{50} values were determined to be 12.09 ± 0.77 and 10.57 ± 0.74 µM for MR766 and Paraiba_01 strains, respectively (Figure 1D,E, Table 2). The antiviral effect of arbidol was further confirmed by immunofluorescence staining; this method revealed the dose-dependent inhibition effect of arbidol on the expression of the ZIKV surface E antigen in Vero cells (Figure 2). The observed anti-ZIKV properties are comparable with those for previously reported small-molecule, such as nucleoside analogues, which exerted anti-ZIKV activities in various cell-based assay systems around the micromolar range (0.2 to 22 µM) [24-27].

Arbidol also showed a significant in vitro antiviral efficacy when tested against two strains of WNV (Eg101 and 13-104). Similarly to ZIKV, the anti-WNV effect of arbidol was most obvious when the compound at concentrations 25 and 50 µM was assayed in WNV-infected HBCA and Vero cells after 48h incubation, respectively, and there was no or negligible antiviral effect seen in virus-infected UKF-NB-6, PS, and Huh-7 cells (Figure 1C). Although, these concentrations of arbidol resulted in the reduction WNV titres 10\textsuperscript{3}-fold compared to non-treated cells, they did not lead to a complete inhibition of virus replication in cell culture (Figure 1C). The incomplete inhibition of viral growth was reflected on the EC\textsubscript{50} values (18.78 ± 0.21 and 19.16 ± 0.29 µM for Eg101 and 13-104, respectively), which were slightly higher (about two-times) compared with those for ZIKV (Figure 1D,E, Table 2). The observed arbidol-mediated decreasing of WNV titre strongly correlated with dose-dependent inhibition of viral surface E antigen expression in the compound-treated Vero cell culture (Figure 2).

Finally, HBCA and Vero cells were also proven suitable to assess the inhibitory effect of arbidol on TBEV (strain Hypr) (Figure 1C). Arbidol inhibited TBEV in dose-dependent manners with an EC\textsubscript{50} value of 18.67 ± 0.15 µM (Figure 1D,E, Table 2). Similarly to WNV, the highest applied concentration (25 µM for HBCA and 50 µM for Vero cells) resulted in an incomplete inhibition of TBEV replication in cell culture, although the viral titre was reduced by 10\textsuperscript{3}-fold compared with non-treated cells (Figure 1C, Figure 2).

Our data are in agreement with numerous reports on in vitro inhibitory activities of arbidol against other viruses of medical interest. It inhibits replication of various subtypes of
human influenza A and B viruses with EC$_{50}$ values ranging from 3 to 9 µg/mL [28]. Arbidol also shows in vitro antiviral effect on Chikungunya virus replication in Vero cells or primary human fibroblasts with EC$_{50}$ values < 10 µg/mL [29] and on Crimean-Congo haemorrhagic fever virus with EC$_{50}$ of 2.8 µg/mL [30]. Pre-treatment of human hepatocellular carcinoma cells Huh 7.5.1 with 15 µM of arbidol for 24h to 48h inhibited HCV replication by up to 10$^3$-fold [11]. Indeed, arbidol exhibited a broad range antiviral effect against respiratory syncytial virus, hepatitis B virus, adenovirus, parainfluenza virus, avian coronavirus, coxackie B3 virus and many other viruses, indicating [11]. Beside in vitro studies, arbidol exerted also substantial antiviral effect in various animal models and was highly effective in clinical trials for prevention and treatment of influenza [31]. Recently, arbidol was reported to induce no embryo toxicity in pregnant female rats, nor to alter the reproductive function of animals, over a 20 day oral administration period of 500 mg/kg doses [32]. Based on these results we can speculate that arbidol might be a promising candidate for effective treatment of viral infections during pregnancy in humans, which could be relevant particularly for pregnant ZIKV patients.

4. Conclusion

In conclusion, we demonstrated that arbidol show a substantial in vitro antiviral activity against ZIKV, WNV and TBEV with EC$_{50}$ values ranging from 10.57 ± 0.74 to 19.16 ± 0.29 µM. The observed antiviral effect is strongly cell-type dependent being obvious only in HBCA and Vero cells, which could be ascribed to differences in compound up-take or metabolic processing of arbidol by individual cell lines tested. Arbidol exerts also significant differences in cytotoxicity profiles when tested in various cell lines; this compound is the most toxic for human hepatoma cells Huh-7, whereas the lowest cytotoxicity was observed for Vero cells. Promising data obtained by our in vitro antiviral screens open the possibility for future testing of antiviral efficacy of arbidol in animal models.

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Authors’ contributions

DR, LE, NT and SK designed and conceived this study. JH, MS and MF performed the experiments. IR and ZH contributed new reagents/analytic tools. LE, DR and NT wrote the manuscript. All authors read and approved the manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Figures
Figure 1
(A) Structure of arbidol. (B) Cytotoxicity of arbidol for Huh-7, PS, UKF-NB-6, HBCA, and Vero cells within the compound concentration range 0-100 μM at 48h post infection. (C) Antiviral effect of arbidol for ZIKV, WNV and TBEV on different cell lines. As arbidol caused different cytotoxicity for individual cell types, the indicated cell lines were treated with different compound concentrations (12.5 μM for Huh-7, 25 μM for HBCA and PS, 30 μM for UKF-NB-6, and 50 μM for Vero) and infected 24h later with the particular virus. Culture supernatants were collected 48h post infection and the viral titres were determined by plaque assay. (D) Dose-dependent effect of arbidol on virus titres at 48h post infection on Vero cell culture. The horizontal dashed line indicates the minimum detectable threshold of $1.44 \log_{10}$ PFU/mL. (E) Inhibition curves of indicated flaviviruses in the presence of a serial dilution of arbidol.
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.

**Figure 2**
Table 1 Cytotoxicity of arbidol for various cell lines of neurone or extraneural origin

<table>
<thead>
<tr>
<th>Cell line</th>
<th>CC$_{50}$ (µM) $^a$</th>
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<tr>
<td>Human brain cortical astrocytes (HBCA)</td>
<td>24.78 ± 0.01</td>
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<tr>
<td>Human neuroblastoma UKF-NB-4 cells</td>
<td>46.99 ± 0.10</td>
</tr>
<tr>
<td>Vero cells</td>
<td>89.72 ± 0.19</td>
</tr>
<tr>
<td>Human hepatocarcinoma cells (Huh-7)</td>
<td>18.69 ± 0.10</td>
</tr>
<tr>
<td>Porcine kidney stable cells (PS)</td>
<td>46.81 ± 1.65</td>
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</table>

$^a$ Determined from three independent experiments.

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

<table>
<thead>
<tr>
<th>Virus</th>
<th>Strain</th>
<th>EC$_{50}$ (µM)$^{a,b}$</th>
<th>CC$_{50}$ (µM)$^{a,c}$</th>
<th>SI (CC$<em>{50}$/EC$</em>{50}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZIKV</td>
<td>MR-766</td>
<td>12.09 ± 0.77</td>
<td>89.72 ± 0.19</td>
<td>7.42</td>
</tr>
<tr>
<td></td>
<td>Paraiba_01</td>
<td>10.57 ± 0.74</td>
<td></td>
<td>8.49</td>
</tr>
<tr>
<td>WNV</td>
<td>Eg101</td>
<td>18.78 ± 0.21</td>
<td></td>
<td>4.78</td>
</tr>
<tr>
<td></td>
<td>13-104</td>
<td>19.16 ± 0.29</td>
<td></td>
<td>4.68</td>
</tr>
<tr>
<td>TBEV</td>
<td>Hypr</td>
<td>18.67 ± 0.15</td>
<td></td>
<td>4.81</td>
</tr>
</tbody>
</table>

$^a$ Determined from three independent experiments.

$^b$ Calculated as a 50% reduction of viral titers using the Reed-Muench method.

$^c$ CC$_{50}$ value determined for Vero cells.
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