Antiviral Activity of Arbidol, a Broad-Spectrum Drug for Use Against Respiratory Viruses, Varies According to Test Conditions

Megan J. Brooks,1 Elena I. Burtseva,2 Philip J. Ellery,3 Glenn A. Marsh,1 Andrew M. Lew,4 Anatoly N. Slepushkin,2 Suzanne M. Crowe,3 and Gregory A. Tannock1,3*

1Department of Biotechnology and Environmental Biology, RMIT University, Bundoora, Victoria, Australia
2DI Ivanovsky Research Institute of Virology, Russian Academy of Medical, Sciences, Moscow, Russian Federation
3Centre for Virology, Burnet Institute, Melbourne, Victoria, Australia
4Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia

The therapeutic activity of arbidol was investigated against representatives of seven different virus families. Its 50% median effective concentration (EC_{50}) was 0.22–11.8 μg/ml (0.41–22 nM). Therapeutic indices of 91 were obtained for type 1 poliovirus and 1.9–8.5 for influenza A and B, human paramyxoviruses, avian infectious bronchitis, and Marek’s disease viruses. Arbidol was more inhibitory for influenza A/Aichi/2/68 (H3N2) virus than rimantadine or amantadine (EC_{50} 10 vs. >15 and >31.6 μg/ml); greater inhibition occurred when end-points were expressed as TCID_{50}s. For respiratory syncytial virus (RSV), a reduction in plaque size but not number was observed. However, when the drug was added to infected cultures (≥5 μg/ml), a 3-log reduction in titer occurred. Arbidol did not inhibit directly influenza A/Aichi/2/68 hemagglutinin (HA) or neuraminidase (NA) activity, but inhibition of fusion between the viral envelope and chicken red blood cells occurred when added at 0.1 μg/ml prior to infection. Arbidol induced changes to viral mRNA synthesis of the PB2, PA, NP, NA, and NS genes in MDCK cultures infected with influenza A/PR/8/34. There was no indirect evidence of enhancement of interferon-α by arbidol following infection with A/Aichi/2/68. Arbidol neither reduced lung viral titers nor caused a significant reduction of lung consolidation in BALB/c mice after administration by the oral and intraperitoneal (i.p.) routes and intranasal challenge with influenza A/Aichi/2/68. A small reduction in lung consolidation, but not viral titer, occurred after i.p. administration and subsequent challenge with RSV. The results indicate the potential of arbidol as a broad-spectrum respiratory antiviral drug.

KEY WORDS: respiratory; antiviral; broad-spectrum; conditions

INTRODUCTION

The compound arbidol (1-methyl-2-[(phenyl-thio)methyl]-indole-3-carboxylate hydrochloride monohydrate) was developed by the Centre for Drug Chemistry (previously known as the All-Russian Chemical-Pharmaceutical Scientific Research Institute), Moscow. Arbidol is an indole derivative, synthesized as an off-white powder from 1,2-dimethyl-5-hydroxyindole-3-acetic acid ethyl ester. It is manufactured in both the Russian Federation and China where it is licensed for use for the prophylaxis and treatment of influenza A and B infections.

Arbidol has been reported to be active clinically against influenza A and B viruses [Glushkov, 1992], respiratory syncytial virus (RSV) [Leneva et al., 2002], and other respiratory viruses [Glushkov, 1992]. Inhibition of influenza A and B virus replication in cell culture has been observed [Fadeeva et al., 1992;...]

Grant sponsor: Australia–Russia Agreement for Collaboration in Medical Research and Public Health (partial support).
Deceased May 28, 2009.
Megan J. Brooks's present address is Department of Epidemiology and Preventive Medicine, School of Epidemiology and Public Health, Monash University, Victoria 3004, Australia.
Glenn A. Marsh’s present address is Australian Animal Health Laboratory, East Geelong, Victoria 3220, Australia.
*Correspondence to: Prof. Gregory A. Tannock, 85 Commercial Road, Melbourne, Victoria 3001, Australia.
E-mail: tannock@burnet.edu.au
Accepted 18 August 2011
DOI 10.1002/jmv.22234
Published online in Wiley Online Library (wileyonlinelibrary.com).
Glushkov et al., 1992; Leneva et al., 1994; Shi et al., 2007), although hemagglutination activity remained unchanged at concentrations that were sufficient to reduce plaque size [Glushkov et al., 1999]. More recently, it has been shown to inhibit hepatitis C virus [Boriskin et al., 2006]. Antiviral activity was considered to be caused by induction of viral lipid envelope fusion of influenza viruses to cell membranes [Fadeeva et al., 1992; Glushkov et al., 1992; Leneva and Hay, 1998]. Arbidol does not interact with viral RNA or DNA by intercalation, which is consistent with later evidence that inhibition takes place at the cell membrane, although other indirect mechanisms involving interaction with DNA/RNA are possible [Glushkov et al., 1992]. Leneva et al. [2002] showed that arbidol inhibited the expression of RSV antigens [Fadeeva et al., 1992; Glushkov et al., 1992; Leneva et al., 1994; Shi et al., 1996].

Fusion of influenza viruses to cell membranes [Boriskin et al., 2006]. Antiviral activity was considered to be caused by inhibition of viral lipid envelope fusion of influenza viruses to cell membranes [Fadeeva et al., 1992; Glushkov et al., 1992; Leneva and Hay, 1998]. Arbidol does not interact with viral RNA or DNA by intercalation, which is consistent with later evidence that inhibition takes place at the cell membrane, although other indirect mechanisms involving interaction with DNA/RNA are possible [Glushkov et al., 1992]. Leneva et al. [2002] showed that arbidol inhibited the expression of RSV antigens [Fadeeva et al., 1992; Glushkov et al., 1992; Leneva et al., 1994; Shi et al., 1996].

**MATERIALS AND METHODS**

**Arbidol**

The compound was generously supplied as a white powder by Academician R.G. Glushkov of the Center for Drug Chemistry, Moscow, Russian Federation.

**Viruses**

Influenza A/Aichi/2/68 (H3N2), a reference prototype strain sensitive to amantadine/rimantadine [Wharton et al., 1994] was obtained from the D.I. Ivanovsky Research Institute of Virology, Moscow, Russian Federation; influenza B/Beijing/184/93 (Dr. A.W. Hampson, WHO Collaborating Centre for Research and Reference on Influenza, Parkville, VIC, Australia); the A-2 strain of RSV (Professor John Mills, Burnet Institute, Melbourne, VIC, Australia); human parainfluenza virus type 3 (HPIV3; Dr. Robert Alexander, Virology Unit, Royal Children’s Hospital, Parkville, VIC, Australia); human parainfluenza virus type 3 (HPIV3; Dr. Robert Alexander, Virology Unit, Royal Children’s Hospital, Parkville, VIC, Australia); poliovirus Type 1 (Sabin); Ms. Margery Kennett, Victorian Infectious Diseases Reference Laboratory, North Melbourne, VIC, Australia); rhinovirus 14 (Dr. Rachel Cameron, Biota Holdings Ltd, Clayton, VIC, Australia)—originally sourced from the European Collection of Cell Cultures, CAMR, Porton Down, Salisbury, Wiltshire, UK; the Australian N1/62 (T) strain of avian infectious bronchitis virus (IBV; Dr. Jagoda Ignatovic, Australian Animal Health Laboratory, Geelong, VIC, Australia); the Australian Woodlands No. 1 strain of Marek’s disease virus (MDV; Marek’s Disease Vaccine Reference Unit, Department of Biotechnology and Environmental Biology, RMIT University, Bundoora, VIC, Australia). Influenza A and B viruses and IBV were egg-grown allantocic preparations. All other viruses were grown in cell cultures.

**Cell Cultures**

The following cell line and primary cultures were used: Madin–Darby Canine kidney (MDCK)—obtained from CSL Ltd., Parkville, VIC, Australia; Hep-2 (human larynx carcinoma) and Vero (African green monkey kidney) lines—Department of Biotechnology and Environmental Biology, RMIT University, Bundoora, VIC, Australia; monkey embryo kidney 3 (MEK3)—Victorian Infectious Diseases Reference Laboratory, North Melbourne, VIC, Australia; Ohio rhinovirus sensitive HeLa—Biota Holdings Ltd., Clayton, VIC, Australia, originally sourced from European Collection of Cell Cultures (ECACC), CAMR, Porton Down, Salisbury, UK.

Primary chicken embryo fibroblasts (CEF) and kidney cultures were prepared from 10- and 18-day old embryos according to the method of Tannock et al. [1985].

**Viral Infectivity Assays**

Influenza and polioviruses. Plaque assays on confluent monolayers of Vero or MDCK cells were performed according to the method of Tannock et al. [1985] for influenza viruses. Cultures were incubated at 34°C in 5% CO₂ for 5 days. Plaque assays for poliovirus were carried out by the same method, except that cells of the MEK3 line and an incubation temperature of 37°C were used. For assays of influenza viruses, crystalline trypsin (final concentration 2 μg/ml) was added to all maintenance media.

**RSV and HPIV.** Plaque assays on monolayers of Vero or HeLa cells for RSV and MDCK cells for HPIV3 were carried out by a modification of the method of Tripp and Anderson [1998].

**Rhinovirus 14.** Infectivity assays were performed by a modification of the Neutral Red release assay described for influenza B viruses [Tannock et al., 1989]. Monolayers of confluent Ohio HeLa cells in 96-well plates were infected with 50 μl of 10-fold virus dilutions in replicates of 8 and adsorption was allowed to take place for 1 hr at room temperature. One hundred microliters of MEM maintenance medium were then added and the cultures incubated at 34°C for 7 days in 5% CO₂. Maintenance medium was then removed and replaced with 200 μl medium/well containing 0.036% Neutral Red. The plates were incubated at 37°C for 30 min. Wells were then washed three times with PBS and 200 μl ethanol/PBS (1:1) added and the plates shaken for 3 min on a mechanical shaker.
shaker to release Neutral Red. Absorbances at 492 nm were measured in an ELISA plate reader. Viral titers, expressed as log$_{10}$ TCID$_{50}$/ml, were calculated by the method of Reed and Muench [1938].

Plaque assays of the Woodlands Number 1 strain of MDV were conducted in secondary CEF [De Laney, 1999]. Plaque assays of the Australian T strain of avian infectious disease virus (IBV) were conducted in primary cultures prepared from the 18-day-old chicken embryo kidneys (CEK) and were conducted according to a method used for the assay of influenza viruses [Tannock et al., 1985].

**Detection of Arbidol-Induced Cytotoxicity in Cell Cultures**

Drug-induced toxicity in uninfected cells exposed to arbidol was assessed by microscopic examination using a modification of the method of Sidwell et al. [1972]. Actively growing cells were seeded to 6-well culture plates and, when confluent, the culture medium was replaced with dilutions of drug in 3 ml maintenance medium. Six serial log- or half-log dilutions of the drug were tested in duplicate. Cell controls contained 3 ml maintenance medium without drug. The monolayers were incubated at 37°C in 5% CO$_2$ over the same time used for assays of individual viruses. Microscopic examination was performed each day to score any cytotoxicity (pyknosis, detachment, shrinkage, granulation, or vacuolation), in comparison with untreated controls. The 50% cytotoxic concentration (CC$_{50}$) was estimated by regression analysis. Cytotoxicity was confirmed by Trypan Blue exclusion counts conducted in parallel.

**Determination of 50% Median Effective Dose (EC$_{50}$) of Arbidol in Cell Cultures**

Plaque assays were performed in the presence of various concentrations of arbidol and viral titers in culture wells exposed to the drug were expressed as a percentage of the untreated virus controls. The concentration of drug that reduced the infectious titer by 50% (EC$_{50}$) was determined by regression analysis [Anonymous, 2007].

**Growth of RSV in Hep-2 Cultures in the Presence of Arbidol**

Confluent monolayers of HEp2 cells in 24-well plates were washed and inoculated with RSV at a multiplicity of infection (MOI) of 0.1. Adsorption was allowed to take place at room temperature for 1 hr and 1 ml of DMEM/F12 maintenance medium containing different concentrations of arbidol was added to different wells and the plates were incubated at 37°C. At different times to 120 hr samples consisting of suspensions of cells and medium from individual wells were prepared and transferred to cryovials which were held at −80°C until assayed.

**Inhibition of Influenza Virus Hemolysis by Arbidol**

At pH 5.0–5.5 the HA glycoprotein of influenza A viruses undergoes a conformational change that exposes the hydrophobic NH$_2$-terminal amino acid residues of the HA2 subunit. During infection, the exposed peptide is inserted into the endosomal membrane at low pH to facilitate fusion between the endosome and the viral envelope. This process can be observed in isolation at the same pH by mixing influenza virus with chicken or guinea pig RBCs by a modification of the method of Plotch et al. [1999]. Different volumes of influenza A/Aichi/2/68 virus (titer 1.5 × 10$^8$ PFU/ml) were mixed with 800 μl of 1% guinea pig RBCs and incubated at 0°C for 30 min. The pH was then lowered to pH 5.0 by adding 100 μl of 0.4 M morpholino ethanesulfonic acid (MES) pH 5.0 and the mixture was incubated for a further 45 min. Tubes were centrifuged and absorbance of the supernatants was measured at 540 nm. The concentration of A/Aichi/2/68 in 100 μl that produced an OD$_{540}$ of 0.5–1.0 was used in inhibition assays.

Mixtures containing increasing concentrations of arbidol were used to test inhibition of fusion. Controls consisted of (a) a no drug (positive) mixture, (b) a no virus (negative) mixture to measure background, (c) a no MES mixture to test for lysis in the absence of MES, and (d) a drug control containing arbidol, but not MES, to confirm that arbidol did not cause RBC lysis. Optical density readings for negative controls were subtracted from test readings. Levels of hemolysis in reactions containing arbidol were expressed as a percentage of the positive control after subtraction of negative control values.

**Preparation and Use of Digoxygenin (DIG)-Labeled Influenza RNA Probes in Northern Blots**

Northern dot-blot hybridization was used to measure levels of influenza A mRNAs in infected cells. RNA probes were labeled by in vitro transcription from DNA clones using a DIG RNA labeling kit (Roche Diagnostics, Mannheim, Germany). Total cell RNA was isolated from infected cells after centrifugation using Trizol$^{	ext{R}}$ reagent (Invitrogen, Mount Waverley, VIC, Australia). Dot-blot hybridization was carried out using protocols from the DIG Application Manual for Filter Hybridization (Roche Diagnostics). One microliter volumes (containing 250 ng/ml of each undiluted sample) were transferred to a positively charged nylon membrane which was air-dried for 30 min and the RNA fixed to the membrane by cross-linking under a UV light for 4 min. The membrane was then incubated at 68°C for 30 min in pre-hybridization buffer, followed by hybridization with 100 ng of each DIG-labeled probe according to the manufacturer’s instructions. Bound probe was detected using alkaline phosphatase-labeled anti-DIG antibody and was visualized by chemiluminescent assay using J. Med. Virol. DOI 10.1002/jmv
CDP-Star and X-ray film (Kodak, Coburg, VIC, Australia) which was developed according to the manufacturer's instructions.

**Studies in BALB/c Mice**

For oral administration, 6 week-old male mice (Animal Resources Centre, Murdoch, Western Australia) were anesthetized with a ketamine/xylazine mixture (final concentrations of 80 and 20 mg/ml in PBS). Mice were held in the upright position and different concentrations of arbidol in 100 µl were administered using a gastric lavage needle attached to a 1 ml syringe. For intraperitoneal (i.p.) administration, 50 µl were administered to unanesthetized mice. For challenge, mice were anesthetized and inoculated i.n. with doses of influenza A/Aichi/2/68 virus or the A2 strain of RSV that produced maximum lung titers within 2–5 days. Virus was recovered from the lungs of individual animals following euthanasia by cervical dislocation. A 10% (w/v) suspension of each lung in DMEM/F12 maintenance medium was prepared which was centrifuged at 5,000 g for 15 min and the supernatants stored at −80°C until assayed. All procedures were approved by the RMIT University Animal Ethics Committee, a body constituted under the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes in conformity with international standards. A/Aichi/2/68 was not adapted by multiple passage to kill mice; with RSV adaptation was not possible. For each virus, endpoints were measured according to lung yields 3–5 days after inoculation.

**RESULTS**

### Solubility of Arbidol

Arbidol is a white, crystalline powder that was shown to be highly insoluble in water, partially soluble in ethanol and completely soluble in chloroform and in glycerol after warming to 60°C. Solubility was retained at 23–60°C. Neither chloroform nor DMSO are suitable for human administration. Chloroform is highly toxic to cells and animals while DMSO has been reported to produce dermatological side-effects that are incompatible with human use [Merck, 1996]. In the present study glycerol was shown to be completely non-toxic to all primary cells and continuous lines used. Arbidol was also found to be partially soluble in polyethylene glycol 4000 and was soluble in glycerol at a stock concentration of 50 mg/ml and could be further diluted in filter-sterilized water or cell culture media. All results reported below were obtained from glycerol preparations.

### In Vitro Therapeutic Activity of Arbidol Against Representatives of Seven Virus Families

Table I shows the CC50, EC50, and therapeutic indices of arbidol in different cell lines and primary cultures for viruses from seven virus families. Similar levels of inhibition have been reported for influenza A and B viruses, RSV, and other respiratory viruses [Glushkov, 1992; Leneva et al., 2002]. For RSV, arbidol caused a significant reduction in plaque size but no reduction in plaque numbers (Fig. 1). The EC50 for MDV (3.9), an oncogenic avian herpesvirus, was comparable with that of the other viruses, except poliovirus type-1. Therapeutic indices of 1.9–8.5 were observed for influenza A/Aichi/2/68 virus (H3N2), influenza B/Beijing/184/93 virus, HPIV3, rhinovirus-14, and IBV and 91 for poliovirus-1. The therapeutic indices of arbidol for RSV and MDV were not determined.

### EC50 of Arbidol for Influenza A and B Viruses in MDCK Culture

In separate experiments the EC50s of arbidol for influenza A/Mem Bel, A/Queensland/6/72, A/Sydney/5/97, and A/Moscow/16/98 (all H3N2 viruses) were 3.7, 5.9, 8.1, and 6.5 (H3N2 viruses) and, for A/PR/8/34 and for A/Moscow/17/98 (H1N1 viruses), 3.6 and 9.0 µg/ml, respectively (68–221 nM for all influenza viruses).

<table>
<thead>
<tr>
<th>Virus family</th>
<th>Representative virus</th>
<th>Susceptible cell culture</th>
<th>CC50 a</th>
<th>EC50 b</th>
<th>Therapeutic index c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orthomyxoviridae</td>
<td>A/Aichi/2/68 (H3N2)</td>
<td>MDCK</td>
<td>17</td>
<td>6.7</td>
<td>2.5</td>
</tr>
<tr>
<td>Orthomyxoviridae</td>
<td>B/Beijing/184/93</td>
<td>MDCK</td>
<td>17</td>
<td>7.1</td>
<td>2.4</td>
</tr>
<tr>
<td>Pneumoviridae</td>
<td>RSV A-2</td>
<td>HEP 2</td>
<td>19</td>
<td>—</td>
<td>d</td>
</tr>
<tr>
<td>Paramyxoviridae</td>
<td>PIV3</td>
<td>MDCK</td>
<td>17</td>
<td>4.9</td>
<td>3.5</td>
</tr>
<tr>
<td>Picornaviridae</td>
<td>poliovirus 1</td>
<td>MEK3</td>
<td>20</td>
<td>0.22</td>
<td>91</td>
</tr>
<tr>
<td>Picornaviridae</td>
<td>rhinovirus 14</td>
<td>HeLa</td>
<td>12</td>
<td>6.5</td>
<td>1.9</td>
</tr>
<tr>
<td>Coronavirusidae</td>
<td>IBV</td>
<td>CEK</td>
<td>100</td>
<td>11.8</td>
<td>8.5</td>
</tr>
<tr>
<td>Herpesviridae</td>
<td>MDV</td>
<td>CEF</td>
<td>ND*</td>
<td>3.93</td>
<td>NA f</td>
</tr>
</tbody>
</table>

A/Mem Bel, an H3N2 reassortant of A/Memphis/1/71 and A/Bellamy/42; A/Queensland/6/72 (H3N2); A/Sydney/5/97 (H3N2); A/Moscow/16/98 (H3N2); A/PR/8/34 (H1N1); and A/Moscow/17/98 (H1N1) were examined in later experiments. Their EC50s were 3.7, 5.9, 8.1, 6.5 (H3N2 viruses) and, for A/PR/8/34 and for A/Moscow/17/98 (H1N1 viruses), 3.6 and 9.0 µg/ml, respectively.

*50% cytotoxic concentration (µg/ml).
*50% median effective dose (µg/ml), defined as the concentration of drug that reduced the infectious titer by 50%.
*EC50/CC50 ratio.
*Reduction in plaque size but not number; unable to estimate therapeutic index.
*Not done.
*Not applicable.
Antiviral Activity of Arbidol

Comparison of Inhibitory Activity of Arbidol, Rimantadine, and Amantadine From Assays in MDCK Cultures

No reduction in plaque numbers occurred in the presence of amantadine or rimantadine (Fig. 2A). There was a 0.5–1.0 log10 decrease in virus titer in the presence of ≥10 μg/ml (≥18 nM) arbidol. Figure 2A also indicates that the EC50 for arbidol, rimantadine and amantadine were 10, >15, and 31.6 μg/ml, respectively. Figure 2B shows results for quantal assays of A/Aichi/2/68 conducted in the presence of different concentrations of the same drugs in liquid maintenance medium (endpoints scored according to the presence of CPE and expressed as TCID50).

A reduction in titer of 1.5 log10 was obtained with arbidol (10 μg/ml) and approximately 1.0 log10 for rimantadine (≥10 μg/ml; ≥56 nM) but no reduction was observed for amantadine (0–30 μg/ml; 56–168 nM). Figure 2A,B indicates that, for A/Aichi/2/68, antiviral activity can be more readily detected in assays where test drugs are present in liquid maintenance medium than the semi-solid medium used for plaque assays, and that arbidol is more inhibitory than rimantadine and both are superior to amantadine. An earlier report also indicated that amantadine is less effective than rimantadine as an inhibitor of influenza A viruses [Browne et al., 1983].

Time of Inhibition of Influenza Replication in Cell Culture by Arbidol

Near-confluent monolayers of the MDCK line were inoculated with influenza A/Aichi/2/68 virus at an MOI of 0.1 in 24-well plates. After adsorption for 30 min at room temperature on a rocking platform, maintenance medium was added to each well. Arbidol (5 or 10 μg/ml) was added at different times p.i. and incubation at 34°C was continued to 80 hr. No differences could be detected in the virus yields from cultures administered either concentration after infection. MDCK cultures were then pretreated with arbidol (10 μg/ml) for different times and then infected with A/Aichi/2/68 under the same conditions. Mean titers ± s.d. for replicate assays at 80 hr for cultures pretreated for 1, 2.5, and 7 hr were 5.70 ± 0.0 (1 hr), 5.57 ± 0.26 (2.5 hr), and 6.04 ± 0.65 (7 hr) PFU/ml, compared with 6.34 ± 0.05, 6.04 ± 0.31, and 5.66 ± 0.90, respectively, for drug-free controls that were administered PBS over the same times (Student’s t-test, P < 0.01 at 1 hr for a 4.31-fold decrease in titer).

Assessment of Inhibition by Arbidol of A/Aichi/2/68-Specific Hemagglutination and Neuraminidase Activity

In a preliminary experiment it was shown that arbidol does not agglutinate chicken red blood cells (0.5%) at concentrations to 250 μg/ml in the absence of virus. Standard hemagglutination inhibition assays [Palmer et al., 1975] were then performed in which mixtures containing increasing dilutions of arbidol and four HA units of influenza A/Aichi/2/68 were incubated for 30 min at room temperature in microtiter plates. One hundred microliters of a chicken red cell suspension were then added and all wells were examined for HA activity after a further 30 min. No inhibition of HA activity was observed at arbidol concentrations of 0.24–250 μg/ml. Similarly, arbidol did not inhibit the neuraminidase activity of influenza A/Aichi/2/68 when tested over the same range by the method of Fiszon and Hannoun [1990].

Inhibition of Viral Fusion by Arbidol From Measurements of Hemolysis of Guinea Pig RBCs

The results of three separate experiments (Fig. 3) indicate that 8% and 6% inhibition of hemolysis
occurred in the presence of arbidol at concentrations of 0.1 and 1.0 \( \mu \text{g/ml} \) \( (P < 0.05) \). At higher concentrations inhibition could not be detected.

Comparison of Growth of A/Aichi/2/68 in VERO and MDCK Cells in the Presence of Arbidol

An indirect method was used to indicate whether arbidol could stimulate IFN-\( \alpha \), the growth of influenza A/Aichi/2/68 virus was studied in both the MDCK and the VERO continuous cell lines in the presence of the drug. The VERO line lacks the IFN-\( \alpha \) genes due to deletions [Diaz et al., 1988; Egorov et al., 1998] and has been used in studies to confirm that the NS1 gene of influenza A viruses is an interferon antagonist [Garcia-Sastre et al., 1998]. If the antiviral activity of arbidol is caused only by its capacity to induce IFN-\( \alpha \), then inhibition of influenza virus in the VERO line should not occur. Six-well plates containing near-confluent monolayers of cells of either the MDCK or VERO line were infected with influenza A/Aichi/2/68 virus at an MOI of 0.01 and maintained in maintenance medium in the presence or absence of arbidol (7.5 \( \mu \text{g/ml} \)). Cell-supernatant suspensions were prepared from duplicate wells at different times after infection to 48 hr and each sample was assayed in duplicate.

Figure 4A shows that a reduction in growth of A/Aichi/2/68 in MDCK cultures (42%) occurred in the presence of arbidol (Student’s \( t \)-test, \( P < 0.01 \)). Figure 4B demonstrates the growth of A/Aichi/2/68 in VERO cells both in the presence and absence of arbidol. By 24 and 48 hr, reductions of 41 and >99% occurred \( (P < 0.01) \). Arbidol inhibited the growth of influenza A/Aichi/2/68 virus in the VERO line and, therefore, its inhibitory activity appears unrelated to its capacity to stimulate IFN-\( \alpha \).

Northern Blot Analysis of Viral RNA Transcription in A/PR/8/34-Infected MDCK Cultures in the Presence of Arbidol

Viral mRNA levels induced during growth in the presence of arbidol were examined by northern dot blot analysis. Near-confluent cell monolayers of the MDCK line in 6-well plates were infected at an MOI of 1.0 and adsorption allowed to take place for 30 min at RT on a rocking platform. MEM Maintenance Medium containing 0 or 10 \( \mu \text{g/ml} \) arbidol was added to each well and the cultures were incubated at 34°C for 48 hr. Cell-supernatant suspensions from individual wells were taken at different times to 48 hr and total RNA extracts of each were prepared. Dilutions of each RNA sample were spotted across a nylon membrane for analysis by northern dot blot. The results of all hybridizations in a representative experiment (one of two performed) are shown in Figure 5 and hybridization against different probes in undiluted extracts is summarized in Table II. Expression of the PB1 and the HA genes over the period did not vary when the virus was grown in the presence of arbidol. After 10 hr, expression of the PB2, NP, NA, M, and NS genes was slightly lower in treated cultures but expression of the PA gene had increased in comparison with untreated cultures. From 24 to 48 hr this trend continued with increased expression of the PB2, PA, NP, NA, and NS genes in treated cultures. The M gene continued to show slightly decreased expression in treated cultures at all sampling times to 48 hr.

Growth of RSV in the Presence of Arbidol

Antiviral activity of arbidol against RSV was investigated in growth experiments in which different concentrations of the drug were added to liquid maintenance medium. A dose-dependent inhibition of growth was observed in the presence of arbidol.
(Fig. 6). After 120 hr a reduction in titer of 3.73 log$_{10}$ PFU was observed at the highest drug concentration tested (10 µg/ml). Comparable inhibition was noted in a further pair of cultures that were infected with an inoculum containing 10 µg/ml and then washed twice with PBS to remove residual drug. These results suggest that the reduction in RSV plaque size by arbidol (Fig. 1) was not caused by inhibition of cell growth.

EVALUATION OF ANTIVIRAL ACTIVITY OF ARBIDOL AGAINST INFLUENZA A/AICHI/2/68 (H3N2) VIRUS AND RSV (A-2 STRAIN) IN BALB/c MICE

Influenza A/Aichi/2/68 Virus. Groups of mice were administered graded doses of arbidol (prepared by dilution of a 50 mg/ml glycerol stock in PBS) twice daily (b.i.d.) for 4 days by the oral and i.p. routes. Treatment was administered on days −1, 0, 1, 2, 3, and 4. After 2 days four mice from each group, except the negative controls, were challenged under anesthesia with 10$_{4.5}$ PFU of A/Aichi/2/68 in 50 µl PBS. Treatment with arbidol was continued and, after 2 days, all animals were killed by cervical dislocation and lung homogenates from individual animals (10% w/v in PBS) were prepared and held at −80°C until assayed by the plaque technique.

The results (Fig. 7A,B) indicate that arbidol orally administered at doses of 2–20 mg/kg b.i.d. did not cause a reduction in lung titers after challenge with 10$_{4.5}$ PFU of A/Aichi/2/68, and no reduction was detected after i.p. administration at doses of 10 and 50 mg/kg. In a separate experiment (Fig. 7C) the mean lung weights of groups administered arbidol or PBS by the i.p. route over the same time were measured. Differences between the Mock control and positive challenge were significant (Student’s t-test, $P < 0.05$) but differences in weight could not be detected between the positive control group and the groups administered arbidol at either dose. However, the mean diameters of plaques from assays of lung extracts of mice treated with either dose were ~50% of those from the controls, indicating some in vivo inhibitory activity.
Sera were prepared from the remaining four mice per group after 20 days and tested for hemagglutination-inhibition antibody against A/Aichi/2/68. Geometric mean titers for each treated group were 50.4–160, compared with 80 for the control group. No dose-dependent enhancement or inhibition of antibody titer was apparent.

RSV A-2 Strain

Groups of four 6-week-old BALB/c mice were administered arbidol by the i.p. route b.i.d. (the same times used for influenza A/Aichi/2/68). All groups, except the Mock control, were challenged at day 2 by the i.n. route under anesthesia with 10^6.2 PFU of the A-2 strain. All mice were sacrificed at day 7 and the lungs were removed, scored for consolidation, weighed, and tested the presence of infectious virus by the plaque technique. The results from Figure 7 indicate that differences in infectious titer between the positive control group and the group administered arbidol by the i.p. route at 10 or 50 mg/kg were present but were not significant. Consolidation of lung tissue, scored visually, was much lower in groups receiving either dose, compared with the unchallenged controls, again indicating some inhibitory activity. Mean lung weights for the positive control group were 67% higher than for the Mock control group. Mean lung weights for groups treated with 10 and 50 mg/kg were 20% and 26% lower than for the controls but these differences were not significant.

DISCUSSION

The neuraminidase inhibitors oseltamivir and zanamivir have allowed significant improvements to clinical management if administered within 48 hr of the commencement of an influenza virus infection [Kim et al., 1997; Hayden et al., 1999]. However, concerns have been raised in recent years about an increase in the incidence of oseltamivir resistance to seasonal H1N1 influenza A viruses [Kiso et al., 2004; Ison et al., 2006; Besselaar et al., 2008; Sheu et al., 2008]. The unavailability of simple, rapid, and sensitive diagnostic kits that distinguish between influenza and other respiratory viruses further limits their application. A broad-spectrum drug with activity against several major respiratory viruses could overcome many of these problems. Such a role had been envisaged for interferon [Monto et al., 1986; Portnoy et al., 1988], but results from clinical trials on the prevention of respiratory viral disease in the 1980s were disappointing [Tannock et al., 1988; Wiselka et al., 1991].

### TABLE II. Gene Expression by Influenza A/PR/8/34 in the Presence of Arbidol

<table>
<thead>
<tr>
<th>Gene</th>
<th>Arbidol</th>
<th>0</th>
<th>1</th>
<th>4</th>
<th>10</th>
<th>24</th>
<th>48</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>±</td>
<td>++</td>
<td>±f</td>
</tr>
<tr>
<td>PB1</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>++</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>PA</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>++</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>HA</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>++</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>NP</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>++</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>++</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>NS</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>++</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

*MDCK cultures were infected with A/PR/8/34 at an m.o.i. of 1.0. Cell-medium suspensions were tested.

†Detected by Northern dot-blot hybridization on cell-medium extracts prepared at each time point.

‡Maintenance medium contained 0 (−) or 10 μg/ml (+) of arbidol.

§Hybridization graded according to the intensity of each blot (−, ±, +, ++, +++).

¶Not done.

‖Shaded areas indicate differences in gene expression in the presence or absence of arbidol.
Based on the present study, arbidol appears a suitable candidate for further investigation as a broad-spectrum respiratory antiviral. Earlier reports indicated that it inhibits the growth of influenza A and B viruses, RSV, and other respiratory viruses in cell culture (Table I) [Fadeeva et al., 1992; Glushkov et al., 1992; Leneva et al., 1994, 2002; Guskova and Glushkov, 1999]. Clinical studies demonstrated protection against influenza A virus infections with reductions in fever, duration of symptoms, and post-influenza complications [Glushkov, 1992; Gagarinova et al., 1993; Glushkov et al., 1999]. Arbidol is widely used in the Russian Federation for the treatment of respiratory illness and has been reported to be well tolerated [Gagarinova et al., 1993; Glushkov et al., 1999].

In the present study cytotoxic activity, expressed as the CC50, in several types of cell culture (2–100 μg/ml; Table I) was similar to that observed for rimantadine and ribavirin [Smee et al., 2002]. Antiviral activity, expressed as the EC50, for influenza A and B viruses, RSV, HPIV3, rhinovirus 14, poliovirus, IBV, and MDV, was 0.22–11.8 μg/ml (Table I). Unfortunately, very few antiviral agents are available for viruses that not members of the Orthomyxoviridae and Herpesviridae and reference substances that allow comparison with representatives of all virus families in Table I simply do not exist. On a molar basis, inhibition of influenza viruses by arbidol was shown to occur at concentrations >10-fold higher than those reported for neuraminidase inhibitors (Table I) [Gubareva et al., 1998; Mendel et al., 1998; Sidwell et al., 1998; Smee et al., 2001]. Arbidol was more active than amantadine and rimantadine (both registered for use in the US and other countries but, unlike arbidol, only active against influenza A viruses) when tested by plaque inhibition (Fig. 2A). However, these differences were greater when the drugs were incorporated in liquid maintenance medium and each endpoint was expressed as a TCID50 (Fig. 2B). Plaque numbers from assays of RSV conducted in the presence of arbidol were not reduced and, therefore, an EC50 could not be estimated although plaques were smaller (Fig. 1). However, growth studies conducted in the presence of arbidol concentrations of 1–10 μg/ml in liquid maintenance medium showed dose-dependent reductions in yield of up to 3.73 log10 PFU (Fig. 6). Clearly, the availability of drug under different conditions of culture affects the EC50 of highly cell-associated viruses such as RSV, and differences were also noted for influenza A/Chic/2/68 virus in MDCK cultures maintained under liquid or semi-solid media (Fig. 2).
Previous reports have suggested that arbidol inhibits influenza growth by blocking fusion between the viral lipid envelope and endosomal membrane at the commencement of infection [Fadeeva et al., 1992; Glushkov et al., 1992]. More recent evidence [Tessier et al., 2011] suggests that arbidol enhances the interaction of virus glycoproteins with the host cell membrane, resulting in inhibition of the conformational change required for viral fusion. However, the very high therapeutic index of poliovirus 1, which lacks a membrane, suggests that other interactions may also be involved [Table I]. Figure 5 and Table II suggest that arbidol inhibits influenza virus entry at the fusion stage and virus egress because viral RNAs accumulate in cells at later stages of infection. Arbidol did not directly inhibit the HA or NA activity of influenza A/Aichi/2/68 virus when tested in vitro, suggesting that inhibition of infectivity occurs by a mechanism that does not involve interference with binding of the virus to sialic acid receptors. MDCK growth experiments indicated that arbidol must be present for 1 hr before virus adsorption in order to inhibit fusion between the viral envelope of influenza A/Aichi/2/68 virus and RBCs. Inhibition only occurred at low concentrations (Fig. 3) which suggests that the interaction is stoichiometrically complex, and possibly a consequence of its solubilization in glycerol. Addition of the drug after adsorption had no effect on the final yields. We were unable to confirm earlier evidence that arbidol is an inducer of IFN-α by a different test [Glushkov, 1992; Glushkov et al., 1992, 1999]. Analysis of viral mRNAs induced during the growth of A/PR/8/34 in the presence of arbidol showed that the PB2, PA, NP, NA, and NS genes were upregulated (Table II). Several influenza gene products (NS1, NS2, PB1-F2) are multi-functional proteins with largely undefined and possibly interactive roles in the regulation of replication. Upregulation of the NS is consistent with a role for IFN-α induction but how changes in the transcription patterns of other genes inhibit fusion is unclear. Other evidence indicates that arbidol does not induce IFN-α gene expression against HCV [Boriskin et al., 2006].

The sequence of events that take place in the very early stages of replication of many viruses is not well understood. Viruses from different families utilize different cell surface proteins with diverse biological functions for attachment and entry to cells. Arbidol inhibits the replication of both enveloped and non-enveloped viruses and is, therefore, unlikely to act as a specific receptor or co-receptor antagonist. It may inhibit some step common to all viruses, such as the exposure of buried hydrophobic regions of the viral attachment proteins that facilitate fusion of membranes [Helenius, 2007]. Further studies using confocal microscopy of cell membranes during early stages of infection in the presence of arbidol and the concurrent use of microarray analysis may provide further insights into these events. A recent study of arbidol-resistant mutants of influenza A/chicken/Germany/27 (H7N7) has identified HA as the major determinant of arbidol sensitivity [Leneva et al., 2009]. Resistance is related to single amino acid substitutions within the HA2 subunit, which stabilize a critical early uncoating step involving acidification [Hay et al., 1986].

BALB/c mice were used to evaluate further the potential of arbidol as an antiviral drug for use against respiratory viruses. Arbidol did not cause reductions in lung titers of mice infected with A/Aichi/2/68 after administration by any route, or infected with RSV after administration by the i.p. route (Figs. 6 and 7). However, for RSV, there was a dose-dependent reduction in the weights of lungs of infected mice treated with arbidol at doses of 20–50 mg/kg b.i.d. (Fig. 7). In a recent study [Shi et al., 2007], BALB/c mice were administered arbidol dissolved in DMSO by the oral route and challenged with a mouse-adapted preparation of A/PR/8/34. Reductions in lung titers were noted at 25–100 mg/kg/day and in lung weights at 50–100/day. Factors, such as the bioavailability of a drug in different solvents and the use of mouse-adapted and non-adapted influenza A viruses with an uncertain passage history need to be taken into account when comparing results from different laboratories. Similar considerations apply to the BALB/c challenge model in the extrapolation of results obtained for arbidol to humans. Some indication of these differences has been shown for oseltamivir, where the oral bioavailability for mice was shown to be 30%, compared with 73% for dogs and 80% for humans [Li et al., 1998; McNicholl and McNicholl, 2001].

ACKNOWLEDGMENTS

This work was undertaken in part fulfillment of requirements for the award of a PhD at RMIT university by the senior author. We gratefully acknowledge the contribution to this work of The Victorian Operational Infrastructure Support Program. We thank Ms. Hyunsuh Kim of the Burnet Institute for assistance in the preparation of the figures and tables.

REFERENCES


De Laney DB. 1999. The development of a serotype 1 vaccine against Marek’s disease. Melbourne, Australia: Department of Biotechnology and Environmental Biology, RMIT University.
Antiviral Activity of Arbidol


