Russian Arbidol Testing Protocol

1. Arbidol substance
Our Institute and the Russian company "Pharmstandard" is the current Arbidol patent holder.

2. Suitable solvent.
We normally dissolve 5 μg of Arbidol to completion in 0.5 ml of 96-proof ethanol at 37^{O}C for 30 min. followed by addition of 4.5 ml of sterile distilled water. This makes a stock solution. For each experiment, a freshly prepared stock of Arbidol is used. **It is very important to make up Arbidol this way for in vitro study.**

3. Cytotoxicity.
To determine drug toxicity in cell cultures we use the visual observation test, increase in neutral red dye uptake and formazan (XTT) method. In our hands, the maximum non-cytotoxic concentration of our arbidol substance in MDCK cells is 30 μg per ml, and the 50% cytotoxic concentration, CC50, is 45 μg per ml.

4. Assay of antiviral activity

To study the antiviral activity of Arbidol, a 96-well antiviral ELISA – based assay is being used. In several studies we showed that it is less subjective, rapid and suitable method to study the effect of Arbidol on virus reproduction (Belshe et al., 1988; Grambas and Hay, 1992). This assay detected expression of viral HA, NP, M proteins on infected cells. Briefly, MDCK cells were seeded in 96-well plates at 3000 cells per well in MEM containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 100 μg/ml kanamycin sulfate. Cells were incubated at 37^{o}C with 5% CO_2 until 90% cell confluency was reached and washed twice with serum-free MEM before infection. Each microtiter plate included uninfected control cultures, virus-infected controls and virus-infected cultures to which ARB was added. The cultures were overlaid with MEM (100 μl) containing 2.5 μg/ml N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) - treated trypsin and various ARB concentrations. After incubation for 30 min - 2 hours at 37^{o}C, 100 μl of virus - containing allantoic fluid (approximately 0.1 PFU/cell) was added to all wells, except uninfected control cells. After incubation for 18 h at 37^{o}C in a humidified atmosphere of 5% CO_2 cells were washed and fixed by adding 50 μl of cold 80% acetone in PBS (20 min.) and well air-dried (no less than 1 hour). Then, the microplates were washed off 3 times with PBS containing 0.05% Tween-20 (TBS). These and all subsequent washes were carried out using the TBS. Next, 100 μl of ELISA buffer (PBS with 1% of fetal calf serum and 0.05% Tween-20) was added to cells followed by microplate incubation at 37^{o}C for 30 minutes. The ELISA buffer was aspirated, and antibodies to NP and M proteins (CDC, 10^{-3} dilution made in ELISA buffer), for influenza A and B viruses were added into wells. After one hr incubation at 37^{o}C and subsequent three-time washes with TBS the wells were fed with 100 μl of horseradish peroxidase – labeled anti-mouse rabbit IgG, 1:1000-diluted, and incubated for another hr at 37^{o}C. After four washes the bound peroxidase was revealed by addition of 100 μl of 0.05% orthophenylendiamine solution (OPD) or 33'55'-tetramethylbenzidine (TMB) in 0.003% citrate buffer, pH 5.0, containing 0.003% hydrogen peroxide. The microplates were kept for 15 – 30 minutes in the dark until the color has developed, and the reaction was stopped by addition of 50 μl of 4N H_2SO_4. Then, the optical density (OD) was measured at 492 nm or 450 nm if OPD or TMD was used, respectively, usinf automated microplate reader. As a control, the mock-infected wells on the same microplate were used.
The percentage inhibition of virus replication by ARB was calculated after correction for the background (cell control) values, as follows: Percent inhibition = 100 x [1-(OD_{450}) of treated sample / (OD_{450}) virus control sample] The IC_{50} value (i.e., the concentration of compound required to inhibit virus replication by 50%) was determined by plotting the percentage inhibition of virus replication as a function of compound concentration.

The most important fact is that arbidol is efficient when it is added at least **30 min - 2 hours (maximum 2-3 hours)** before infection. Addition of arbidol at the same time or after infection does not significantly affect virus yield.

Yours sincerely

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