Arbidol: A Broad-Spectrum Antiviral Compound that Blocks Viral Fusion

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Abstract: Arbidol (ARB; ethyl-6-bromo-4-[(dimethylamino)methyl]-5-hydroxy-1-methyl-2-[(phenylthio)methyl]-indole-3-carboxylate hydrochloride monohydrate), is a Russian-made potent broad-spectrum antiviral with demonstrated activity against a number of enveloped and non-enveloped viruses. ARB is well known in Russia and China, although to a lesser extent in western countries. Unlike other broad-spectrum antivirals, ARB has an established molecular mechanism of action against influenza A and B viruses, which is different from that of available influenza antivirals, and a more recently established mechanism of inhibition of hepatitis C virus (HCV). For both viral infections the anti-viral mechanism involves ARB inhibition of virus-mediated fusion with target membrane and a resulting block of virus entry into target cells. However, ARB inhibition of fusion exploits different ARB modalities in case of influenza viruses or HCV. This review aims to summarize the available evidence of ARB effects against different groups of viruses, also, to compare various aspects of ARB anti-fusion mechanisms against influenza virus and HCV (with reference to different stringency of pH-dependence of these two viral fusogens) and to discuss further prospects for ARB and its improved derivatives of the parent compounds.

Keywords: Arbidol, antiviral, influenza viruses, hepatitis C virus, viral fusion.

1. INTRODUCTION

During the last half - century Virology as a discipline has achieved its goals by defining virus structure and function (replication) and creating means to combat viral infections through vaccine prophylaxis and/or antiviral drug treatment. However, for some viral infections, e.g., HIV or hepatitis C, vaccines are not yet available, while for viruses like influenza virus, with rapidly changing antigenic profile, vaccines must be modified nearly every year to catch up with emerging viral variants. In such instances chemotherapy remains an alternative or the only defense option.

Since viruses utilize host cell metabolism for their own replication, many antiviral agents inevitably impact cellular metabolic pathways or interfere with cellular functions or critical steps in virus-cell interactions. The broad-spectrum antivirals target rate-limiting events in viral replication cycle such as envelope protein glycosylation, processing and folding or viral-cell membrane fusion during viral uncoating or assembly [1]. The other group of virus-specific antivirals target virus-encoded activities (enzymes) like viral polymerase or protease [2, 3], and these agents usually possess high (100 – 1000) therapeutic indices (TI). However, the drawback of their high specificity is a rapid virus adaptation to the drug and eventual development of drug resistance due to accumulating mutations, as exemplified by HIV resistance to antiretroviral therapy. The broad-spectrum antivirals are less prone to developing drug resistance but their efficiency is usually a trade-off between some cytotoxicity and anti-viral effects.

Given the shortcomings with targeted antivirals, therapeutic regimens have in some cases shifted towards complementing enzyme inhibitors with less specific agents like inhibitors of viral fusion or viral co-receptors [4]. Combination therapy by drugs with different modes of action and resistance profiles could be beneficial for a number of viral infections.

While rational structure-based antiviral drug design is expected to provide significant new therapeutic leads, traditional chemical synthesis, possibly reinforced by combinatorial chemistry, will nonetheless reveal novel structures or expand the power of already known compounds as newly emerged broad-spectrum antivirals. This review outlines structure, pharmacokinetics, safety, tolerability, activity *in vitro* and *in vivo* and mechanisms of viral fusion inhibition of arbidol, a broad-spectrum antiviral with excellent potential for adjunct and/or specific clinical therapeutic applications. The immunomodulating and anti-oxidant aspects of ARB action are so far inconclusive [5] and, hence, are left out of the scope of this review.

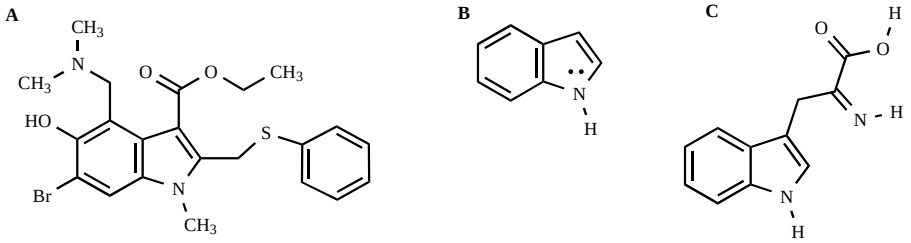

Fig. (1). Structure of arbidol (**A**), indole (**B**), and tryptophan (**C**).

2. ARB STRUCTURE AND SYNTHESIS

Arbidol (ARB; ethyl-6-bromo-4-[(dimethylamino)methyl]-5-hydroxy-1-methyl-2-[(phenylthio)methyl]-indole-3-carboxylate hydrochloride monohydrate) was developed by the Russian Research Chemical Pharmaceutical Institute about 20 years ago [6], and since 1990 ARB has been used as an over-the-counter medicinal drug in Russia, primarily for prophylaxis and treatment of acute respiratory infections including influenza.

The ARB compound is an indole derivative and is synthesized from 1,2-dimethyl-5-hydroxyindole-3-acetic acid ethyl ester (Fig. (**1**)) [7]. Briefly, the ethyl ester is acetylated with acetyl anhydride to produce an O-acyl derivative, which is then brominated to the dibromide compound. In the presence of potassium hydroxide, the dibromide compound is reacted with thiophenol to produce an intermediate, which then undergoes a conventional Mannich conden-

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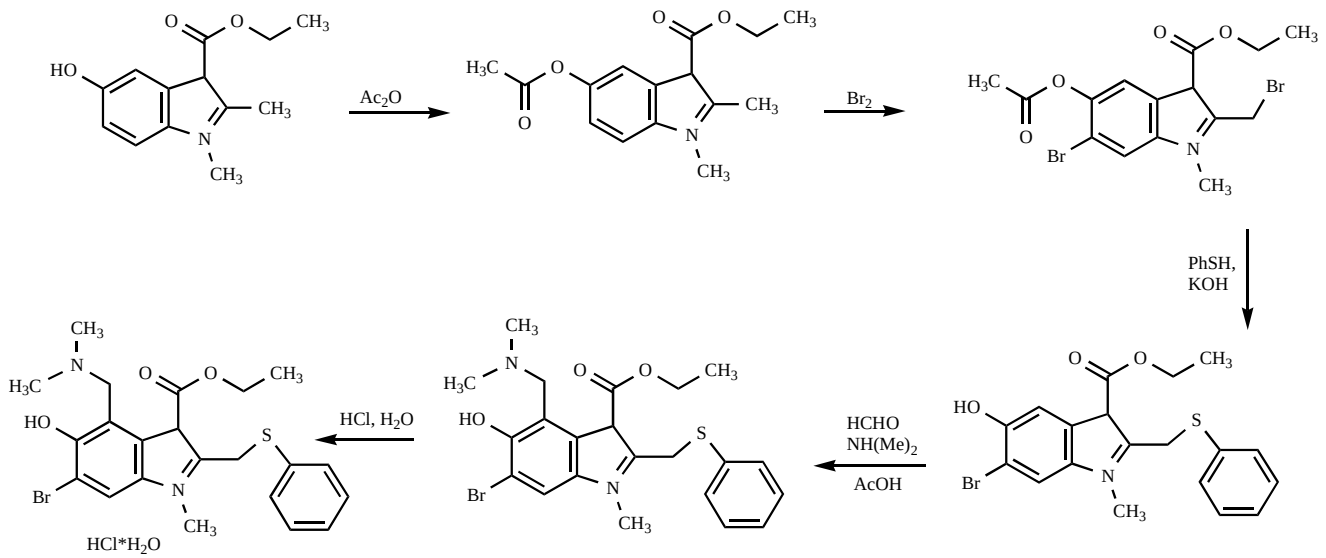

Fig. (2). Synthesis of arbidol.

sation with formaldehyde and dimethylamine in acetic acid. The resulting free base arbidol is then treated with aqueous hydrochloric acid to make the final compound, ethyl ester 6-bromine-5-hydroxy-4-dimethyl-aminomethyl-1-methyl-2-phynylthiomethyl indole-3-carboxylic acid hydrochloride monohydrate (Fig. (**2**)).

Structurally, this compound is a hydrochloride salt with weak base properties (Fig. (**1**)). ARB is likely to be a prodrug with good oral bioavailability and is efficiently hydrolysable in plasma due to the presence of a carboxylic acid ester in its structure. ARB may exist as a zwitterion having one positive charge and one negative charge at neutral pH. If ARB's ester moeity becomes hydrolyzed *in vivo*, ARB could display lysosomal and/or mitochondrial accumulation. As an indole (5-oxyindole) derivative, ARB has an affinity for membrane interfaces, as is the case for tryptophan within membrane proteins [8, 9]. This interfacial preference of indoles is linked to the flat rigid structure of these molecules and their aromaticity which allows an interaction between the indole ring through its pi–electron cloud and the nitrogen of the lipid head group in cell membranes [9, 10]. The ARB molecule becomes more protonated at acidic pH, which enhances its biological activity [11]. As will be shown below, the propensity of ARB for cell membranes is crucial in the context of its anti-HCV action.

3. ARB SAFETY AND TOLERABILITY

ARB has demonstrated low toxicity upon a single-dose oral administration. The animal LD_{50} (50% lethal dose) was 687 mg/kg for mice, 3000 mg/kg for rats and 4000 mg/kg for guinea pigs [5]. Long-term ARB intake in doses up to 100-125 mg/kg in rats (six months), guinea pigs (three months), at 50 mg/kg in rabbits (two months) and at 25 mg/kg in dogs (six months) did not induce pathologic changes in animals as confirmed by clinical, hematological, biochemical and pathological data. It was shown that in therapeutic doses ARB possessed no mutagenic or teratogenic activity. When used in non-toxic doses for pregnant female rats, an ARB dose of 250 mg/kg (a that exceeds a maximum daily human dose by a factor of 20-30) did not affect the embryogenesis and postnatal development of albino rats as indicated by anatomical, histological and physiological tests [5]. The safety and tolerability of ARB had also been evaluated in a placebo-controlled study on 39 healthy volunteers and 22 patients with acute respiratory diseases in the recovery stage, with daily oral administration of ARB in doses ranging from 200 mg to 800 mg for ten days. The study participants were followed up for another 10-15 days. The physical and biochemical examination of main organs and systems did not reveal any significant differences between ARB-treated and control groups indicating good tolerability and safety of ARB in humans [5].

4. ARB PHARMACOKINETICS

ARB pharmacokinetics was studied in rats using HPLC analysis and radiochromic dosimetry (that is, a quantitative image formation technique based on direct color change in response to radiation exposure) with ^{14}C– labeled ARB [12 – 14]. Twenty minutes after oral administration ARB appeared in blood and rapidly distributed in tissues and organs with maximal accumulation in liver (3.1% per one gram of tissue), pituitary gland (1.7%), kidneys (1.2%), lymphatic nodes (1.2%) and thyroid, adrenal gland, bone marrow, lungs, plasma, thymus and spleen (less than 1% each). About 40% of a total intake dose of ARB is excreted unchanged within 48 hours, mainly with feces (38.9%) and much less with urine (0.12%). Similar experiments in mice showed that 90% of ingested ARB is excreted in 48 hours, of which 39% was unchanged and the rest of the drug was comprised of ARB metabolites [5, 7]. In Madin-Darby canine kidney (MDCK) cells ARB undergoes rapid biotransformation reaching its peak concentration at 3-5 hrs post-inoculation. By 24 hrs ARB levels decrease to less than 50% of the input dose suggesting ARB degradation and/or biotransformation to active metabolites [15].

ARB pharmacokinetics was studied in 50 healthy volunteers divided into 5 groups of 10 subjects each, who received orally 50, 100, 200, 500 and 1000 mg of ARB, respectively [16]. All five doses were well tolerated. ARB kinetics were studied using a highly sensitive and selective HPLC technique. The maximal ARB plasma concentration (C_{max}) increased with increasing of intake dose, and for a 200 mg dose it peaked at 1.23 μg/ml on 1.6 – 1.8 hrs after administration. The T_{max} (time to maximum drug concentration) showed no dependence on ARB dose, as well as ARB half-life period that was around 16 -21 hrs [16]. These results were confirmed in an ARB pharmacokinetic study performed on healthy male Chinese volunteers [17].

Since ARB is likely to be an ester prodrug, its antiviral activity may be produced by one or several hydrolyzed metabolites. Indeed, in the urine of mice fed with ARB, up to 12 ARB metabolites have been detected, of which some were oxidized sulfoxide and sulfone forms of ARB. Using thin-layer chromatography, HPLC and mass spectrometry, ARB metabolites were identified and quantified in

chloroform extracts of urine of rats fed with ARB [14]. It was revealed that three metabolites exceeded the content of the unchanged ARB compound. Finally, the main metabolic biotransformations of ARB appeared to be (i) a removal of the alkyl amino group in position 4 of the benzene ring (three metabolites), (ii) sulfur oxidation with formation of sulfides (one metabolite), sulfoxides (two metabolites) and sulfones (two metabolites), (iii) conjugation over the hydroxyl group of the aromatic ring with the formation of corresponding glucuronid and sulfate (two metabolites), as well as glucuronid and sulfate products of ARB demethylation over the dimethylamine group (two metabolites). The main metabolic pathways of ARB are presented on (Fig. (**3**)).

5. ANTI-VIRAL SPECTRUM OF ARB

ARB exhibits a wide range and potent antiviral activity against a number of viruses including influenza A, B and C viruses [18], respiratory syncytial virus [19], adenovirus [20], parainfluenza type 5 and rhinovirus type 14 [21], avian coronavirus, infectious bronchitis virus and Marek disease virus [21], hepatitis B virus [22] and hepatitis C virus [23]. Recently, Chinese investigators confirmed the wide antiviral spectrum of ARB against influenza A, respiratory syncytial virus, human rhinovirus type 14, coxsackie B3 virus and adenovirus type 7 [24]. There are presently no published indications of ARB activity against other medically important viral pathogens like HIV-1, SARS coronavirus or Ebola virus. The details of ARB action against influenza viruses and hepatitis C virus will be presented below. The extensive list of ARB-sensitive viruses encompasses RNA and DNA viruses, enveloped and non-enveloped viruses and pH-dependent and pH-independent viruses emphasizing a broad spectrum of ARB antiviral activity. At the same time, the wide spectrum of ARB's activity suggests that ARB targets common critical step(s) in virus – cell interaction.

6. ARB AND INFLUENZA VIRUSES

6.1. ARB Effect on Influenza Virus: *In vitro* Studies

ARB demonstrated a broad and potent activity against three antigenic serotypes of human influenza A viruses (H1N1, H2N2 and H3N2) [18, 25, 26], human influenza B and C viruses [25] and avian influenza A viruses (H5N1 and H9N2) [27,28,29]. Virological testing was performed in MDCK cells by cytopathic effect inhibition assay, plaque-forming assay and enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies (AB) to viral capsid (NP) and matrix (M1) proteins and secondary peroxidase-labeled AB. Percentage of viral inhibition was determined against uninfected and infected untreated controls followed by calculation of minimal inhibitory concentration 50 (IC_{50}). Comparison of ARB activity (20 μM) to that of other anti-influenza drugs (amantadine, rimantadine, ribamidil, zanamivir and oseltamivir) taken in equimolar concentrations, showed stronger or comparable inhibitory effect on influenza viruses' replication by ARB [29]. ARB's IC_{50} ranged from 3 μg/ml to 12.5 μg/ml. Unlike rimantadine which blocks only rimantadine-sensitive strains of influenza A virus, ARB strongly inhibited both rimantadine-sensitive and rimantadine-resistant strains of influenza A viruses to similar degrees [25]. ARB was shown to be effective in mice infected with A/PR/8/34 (H1N1) or A/Aichi/2/69 (H3N2) influenza A viruses. When administered orally 24 hours before virus exposure and continued for 5 days

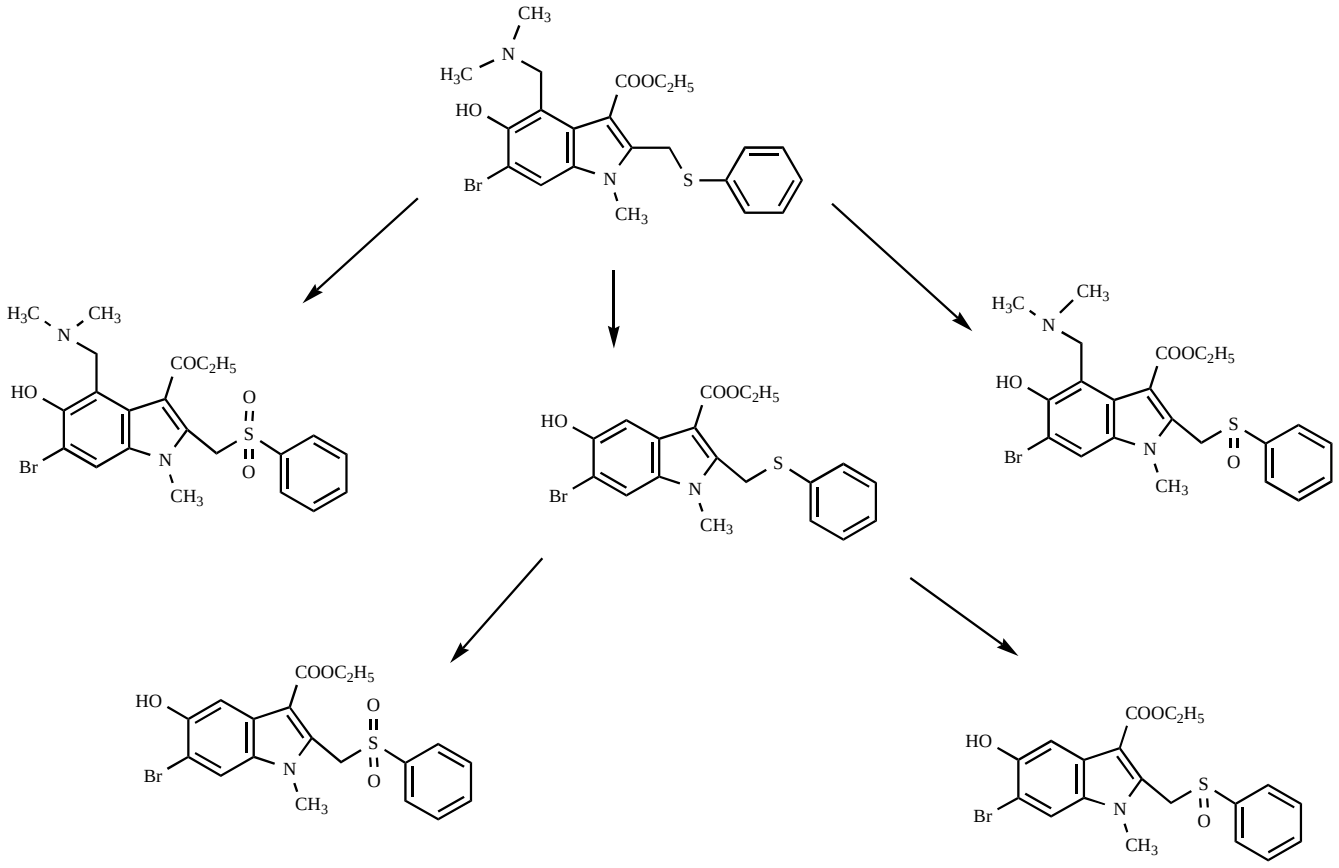

Fig. (3). The main biotransformation pathways of Arbidol.

Removal of alkyl amino group at position 4 of benzene ring.

Sulfur oxidation at position 2 with formation of sulfides , sulfoxides and sulfones.

onwards, ARB (60 - 120 mg/kg/day) significantly reduced the virus titer in lungs as well as mortality rate by 70-80% [5].

6.2. Efficacy of ARB Against Avian Flu (H5N1) Viruses

A highly pathogenic avian influenza virus H5N1 has crossed the species barrier in Asia to cause many human fatalities and poses an increasing pandemic threat. In face of an emerging pandemic, antiviral drugs have enormous potential for preventing deaths of infected persons and the further spread of disease. Therefore, it is of utmost importance to have available data about efficacy of ARB against H5 influenza viruses. We studied the efficacy of ARB in cell culture against different H5 influenza viruses. Although ARB inhibited replication of avian influenza viruses A/Hong Kong/156/97 (H5N1) that caused human infection in Hong Kong in 1997, its inhibitory effect was not as strong as that over human strains of influenza viruses [29]. In case of A/Hong Kong/156/97 (H5N1) virus, the IC_{50} of ARB was 30 μg/ml [29].

ARB also effectively inhibited replication of three H5 viruses isolated in Russia from wild birds. The hemagglutinins (HAs) of these isolates are closely related to the HA of A/Hong Kong/156/97 (H5N1) virus. The susceptibilities of these avian isolates to ARB were within the ranges for human influenza viruses (IC_{50} ranged from 4 μg/ml to 7.5 μg/ml) [27]. ARB also inhibited replication of highly pathogenic avian influenza H5N1 virus isolated from chickens in Novosibirsk, Russia, in which case the IC_{50} of ARB was 9μg/ml and comparable to the IC_{50} of rimantadine and ribavirin tested in parallel [30].

The data on ARB efficacy against H5 influenza virus in cell culture were confirmed in animal experiments. Mice given ARB at 50 or 100 mg per kg of body weight per day, were completely protected against challenge with pathogenic influenza avian A/Kurgan (H5N1) virus isolated during an outbreak of influenza in poultry in Russia (R. Khamitov, personal communication).

6.3. Mechanism of Action of ARB Against Influenza Virus

The influenza virus replication cycle commences when influenza virions adsorb onto the plasma membrane. Bound virions are then trapped in coated pits, internalized by endocytosis where the viral envelope fuses with endosome membranes in an acidic environment. The influenza virus envelope contains two surface proteins, the neuraminidase (NA) and hemagglutinin (HA). ARB does not affect viral NA activity or viral transcription/translation using specific *in vitro* tests, nor does ARB inhibit adsorption of fluorescent probe-labeled virus particles to MDCK cells [31]. At the same time, in a one-cycle infection experiment, one hour pre-treatment with ARB of MDCK cells abolished influenza virus A and B yields by 80% and 70%, respectively, whereas the addition of ARB 30 minutes after infection had no effect [25] suggesting that ARB affects early post-adsorption stages of virus replication with possible involvement of the second surface viral protein, the HA. Using fluorescence dequenching assays ARB was shown to inhibit membrane fusion between the virus and the plasma membrane and also between the virus and endocytic vesicle membranes [31].

Influenza virus HA mediates viral envelope fusion with endosomal membranes. Fusion requires HA conformational changes that occur only at acidic pH; these changes lead to the exposure of the N-terminal fusion peptide contained in the HA2 subunit of HA, permitting its interaction with the target endosomal membrane [32]. To ascertain if ARB targets viral HA, we analyzed a series of viral reassortants between highly- and poorly ARB-sensitive viruses possessing different gene constellation. It was found that only reassortants that acquired HA from ARB-sensitive viruses retained sensitivity to ARB. No other viral gene could be linked to ARB-sensitivity.

To more precisely map ARB sensitivity, we generated seven ARB-resistant viral mutants after 15 serial passages of wild type virus in MDCK cells in the presence of increasing ARB concentrations (from 5 to 20 μg/ml). Nucleotide sequencing mapped all ARB-resistant non-synonymous mutations to the HA2 subunit of HA. Unlike the wild-type HA with fusion optimum at pH 5.0, the ARB-resistant mutants displayed a pH optimum at 5.2 in a virus-specific haemolysis assay. Using a HA conformation-sensitive monoclonal AB it was found that ARB blocked the conformational transition of HA to its fusogenic state without affecting HA conformation of ARB-resistant mutants in infected cells (I. Leneva, A. Hay. 12th International Congress of Virology, Paris, 2002, Abs.1077). The ELISA test using HA conformation-sensitive antibody and *in vitro* hemolysis assay are equivalent to virus-induced fusion in cultured cell but, unlike the latter, are quantitative and more accurate.The CC_{50} (50% cytotoxic concentration) of ARB was shown to be 40-60 μg/mL [18, 26], about 5-fold higher than that required for inhibition of virus growth in cell culture (IC_{50} = 10 μg/ml) and for rendering the low-pH - induced conformational changes of HA.

Therefore, our *in vitro* studies demonstrated that ARB acts by increasing influenza virus HA stability and preventing low pH-induced HA transition to its fusogenic state, thus blocking infection at the viral fusion stage (I. Leneva, A. Hay. 12th International Congress of Virology, Paris, 2002, Abs.1077). Since the three-dimensional structure of influenza virus HA is available, future X-ray crystallography structure analysis will reveal how ARB stabilizes the HA molecule.

6.4. ARB Effect on Influenza Virus: Clinical Experience

In 1990, ARB was approved for treatment and prophylaxis of influenza A and B infection and since then has been widely used in Russia. ARB is manufactured as coated tablets or capsules of 0.1 g for adults or 0.05 g for children. For influenza treatment, the recommended dose is 0.2 g 4 times a day for 3-5 days. For urgent prophylaxis of subjects who are in contact with symptomatic patients, ARB is given in 0.2 g doses once a day for 5-10 days. For influenza prophylaxis during epidemics ARB should be taken in 0.1 g doses twice a week, with 3-4 days' intervals, for 20 days. Laboratory and clinical investigations demonstrated that ARB has low toxicity for animals and is well tolerated by patients. ARB displays oral bioavailability of 38%. In several studies on clinically ill patients, oral ARB intake (200 mg daily for 5-10 days) was shown to reduce the duration of illness by about 1.7 – 2.65 days. ARB also prevented the development of post - influenza complications and lowered the frequency of re-infections in patients affected with influenza [5].

A sound prophylactic effect of ARB had been demonstrated in numerous clinical studies of various sizes [5]. Daily oral administration of 200 mg doses of ARB in pockets of respiratory infections (e.g., families, hospital wards) during the epidemic outbreak of influenza B reduced the number of diseased by 86.3%. During a mixed epidemic community outbreak of influenza A (H3N2) and B the prophylactic ARB effect depended on ARB dose schedule and was the highest at a dose of 100 mg twice weekly. The ARB efficacy index (EI), that is, the ratio of the number of diseased per hundred of subjects taking placebo to that taking the drug, was 3.12. ARB prophylaxis during community outbreaks caused by influenza A H3N2 or H1N1 viruses, was the highest in non-vaccinated (EI=2.5) compared to vaccinated (EI=1.3) subjects. The protective effect of ARB lasted beyond the end of its prophylactic course and was superior to that of rimantadine in this respect. The EI of ARB and rimantadine measured at 10 days after the end of the prophylactic course, was 5.16 and 1.0, respectively [5]. The prophylactic effect of ARB was also studied in a randomized placebo-controlled trial conducted by the Pasteur Institute in St. Petersburg in 1995 on 155 children receiving ARB twice a week for 3 weeks before influenza morbidity had peaked. ARB prophylaxis reduced duration of illness by 1.8-3.5 days, and overall morbidity was reduced by 1.2-4

fold, and was age-dependent (Kubar, O.I.; Stepanova, L.A.; Safonova, L.S.; Rosaeva, N.R. IVth Russian National Congress "Man and Medicine", Moscow, 1997, p. 269).

In an Ukranian study over the winter of 2002/2003, two groups of healthy children aged 6-12 yrs and over 12 yrs received 0.1 g or 0.2 g ARB, respectively, once every 3 days for 4 weeks (total number of children was 156). ARB prophylaxis prevented the development of severe forms of respiratory disease and/or complications [33]. Similar results were obtained on a cohort of 500 children, where ARB prophylaxis led to a milder respiratory infection course and reduced incidence of complications. In children with chronic respiratory infections who were taking ARB, the number of sick subjects was 3.7-fold lower than in the control untreated group, and the number of cases of acute bronchitis, pneumonia or otitis was 4-fold lower than in the control group [34].

In several controlled studies on four groups of students and industrial workers ARB prophylaxis was shown to be 80% effective during influenza A outbreaks in 1988-1989, with ARB optimal dosage of 0.1 g two times a day for 10 – 18 days [35].

Clinical trials have also shown ARB effect for prophylaxis and treatment of influenza in children. One extended study conducted by the Institute of Virology in Moscow, encompassed three epidemic seasons in 1993, 1994 and 1995. A total of 335 children aged 6-15 yrs from closed children collectives received 50 mg of ARB 3 times a week for 5 weeks. Regardless of viral etiology, the cumulative morbidity analysis yielded ARB efficacy index of 2.05 – 2.22 and ARB efficiency coefficient of 51.3-55% [36]. The acute respiratory disease in ARB-treated children took a milder, and 2-3 days shorter course with little, if any, fever, while clinical symptoms were reduced to rhinitis and hyperemic mucous. The incidence of recurrent illness was 4.6-5 times higher in the placebo group than in the ARB-treated group [36].

These results were confirmed in St. Petersburg clinical study on 158 pre-school and school children infected with different influenza virus serotypes and non-influenza respiratory viruses [37]. Treatment efficiency coefficient in ARB-treated children (10 mg per kg weight, four doses during 5 days course) was 84.8%, with statistically significant reduction of fever period, larynxotracheitis symptoms, and virus nasal shedding. The therapeutic efficacy of ARB was most pronounced when the drug was administered early in infection [37].

ARB therapeutic efficacy and safety was confirmed by Chinese investigators in a randomized, double blinded, placebo controlled study on patients presented with fever within 36 hrs after the onset of respiratory disease during the community-acquired influenza outbreak [38]. A total of 125 patients [59 treated with ARB (0.2 g, 3 times a day for 5 days) and 66 placebo controls] with laboratory-confirmed influenza were followed up until completion of the trial. In ARB-treated group the cumulative proportion of patients with alleviated symptoms was significantly higher than in placebo group, with similar frequency of adverse events (gastro-intestinal symptoms and increased transaminase levels) in both groups.

Collectively, clinical experience of ARB application for prophylaxis and treatment of influenza compares favorably with clinical data available for adamantanes and neuraminidase inhibitors [39].

6.5. ARB Resistance

Apart from ARB, two other classes of anti-influenza drugs, adamantanes and neuraminidase inhibitors, are currently available for influenza therapy. However, emergence of resistance to these drugs, particularly to adamantanes, has been detected, which raises concerns regarding their widespread use [40,41]. Rapid, within 1-2 passages, emergence of virus variants resistant to amantadine and rimantadine has been shown in many cell culture or animal studies. In humans, adamantine- resistant viruses could be isolated as soon as 2-4 days after the commencement of treatment [42]. More recent studies of influenza clinical isolates have shown that the incidence of adamantine-resistant influenza A viruses has gone up markedly from 1.9% to staggering 90% in some countries [41] which necessitates the monitoring of drug resistance for naturally circulating influenza A viruses.

In our experience, selection of ARB-resistant mutants required considerably longer time compared with selection of amantadine- and rimantadine-resistant mutants (i.e., 15 passages for ARB-resistant mutants *vs.* only 2-3 passages for amantadine- and rimantadine-resistant mutants) [42]. This could account for the fact that while ARB has been in clinical use for 15 years, the ARB-resistant viruses have not been isolated so far [26]. ARB also inhibited replication of rimantadine-resistant influenza viruses [25]. This latter result is not surprising since rimantadine targets the viral matrix protein. This feature of ARB is of clinical importance given recent reports of 10% - 18% increases in the number of rimantadine-resistant influenza A (H3N2) virus strains in Russia during recent epidemic seasons. [26].

A study of more than 160 clinical isolates of influenza A and B viruses in Russia taken between 2002 and 2005, showed their high sensitivity to ARB including rimantadine-resistant isolates and influenza-like B / Hong Kong viruses. Clinical isolates of influenza B-like viruses, namely, B/Shankhai/361/02, were less sensitive to ARB, but their IC_{50} fell in the range of sensitivity for laboratory and clinical isolates. Therefore, at present, there is no evidence for naturally occurring resistance to ARB in any influenza virus isolates [26].

We addressed the possibility of ARB resistance development in a pilot clinical study on 14 symptomatic patients who were receiving ARB treatment for 5 days during the illness. The influenza A and B viruses were isolated from patients before and at different times after the beginning of treatment followed by determination of their susceptibility to ARB in MDCK cells using ELISA assay. All isolates appeared to be equally sensitive to ARB with IC_{50} falling in the range between 7.0 and 12.5 μg/ml, similarly to IC_{50} previously observed for laboratory and clinical isolates. Two matched pairs of isolates from two patients in whom we were able to obtain the pre-treatment and day 4 or day 5 samples, were chosen for sequence analysis of the HA gene. We did not find any amino acid changes in HA genes that had been previously identified as being involved in ARB resistance (Section 6.3), indicating that ARB resistance is unlikely to occur during 5 days of treatment of acute influenza infection (Leneva, I.A., Burtseva, E.I, Shevchenko, E.I, Shuster, A.M. 46th Interscience Conference on Antimicrobial Agents and Chemotherapy. San Francisco, CA, September 27-30, 2006, p. 463). Similar larger scale studies are now in progress.

7. EFFECTS OF ARB ON HEPATITIS C

Hepatitis C is a global health problem with an estimated 170 millions infections that cause approximately 500,000 deaths per year. Of those acutely infected with HCV, only 20% - 30% can clear infection, with the majority progressing to chronic infection. Chronic infection results in spectrum of liver disease, including chronic hepatitis, cirrhosis and hepatocellular carcinoma, which makes hepatitis C the most frequent indication for liver transplantation in many countries.

Currently, there is no vaccine against hepatitis C, and only limited therapeutic options available in the form of pegylated interferon in combination with ribavirin. Hepatitis C pharmacology lags behind other antivirals because HCV is very difficult to culture, and only recently [43] a recombinant infectious HCV became available for *in vitro* experimentation. Before that, two major *in vitro* models of HCV infection had been available, the replicon [44, 45] and pseudoparticle systems [46].

The replicon system is a non-productive HCV replication system established by transfection of incomplete or complete HCV genome into human hepatoma Huh7 cells. The HCV pseudoparticles are recombinant surrogate viruses containing a retrovirus core coated with the HCV envelope proteins, E1 and E2. Both HCV replicon and pseudoparticles models have been in use for several years, and major developments in molecular biology of HCV, including anti-HCV drug discovery, have been derived from these models. After screening in replicon-based systems, several structure-based, rationally designed small molecule compounds targeting the HCV protease and polymerase are now in different phases of clinical trials.

7.1. Effects of ARB on Chronic HCV Replication

Assuming the broad anti-viral spectrum of ARB, we tested ARB for antiviral activity in a replicon Huh7-derived FL-Neo cell culture harboring an autonomously replicating genomic length genotype 1b HCV replicon. FL-Neo cells were cultured in the presence of sub-toxic ARB concentration for over 12 weeks. As ARB half-life in cultured cells is about 18 hrs, cells were fed with freshly made ARB solution every day. HCV protein expression was monitored by Western blot for structural (core) and non-structural (NS5A) proteins and by quantitative real-time PCR for viral RNA. ARB induced a progressive decline in both viral protein and RNA expression until RNA content dropped to 0.45% of that of untreated FL-Neo cells on the ninth week post-treatment, and became undetectable after 10 weeks of ARB treatment. The FL-Neo cells appeared to be completely cured of HCV, with no HCV rebound in the following two weeks [23].

7.2. Effects of ARB on Acute HCV Infection

The replicon system led to the development of the JFH-1 replicon [43], the first truly infectious HCV culture system to faithfully recapitulate all the components of the HCV life cycle in cell culture: binding, adsorption, replication, assembly, and production of infectious HCV virions. Huh 7.5.1 cells (a clonal line derived from Huh7 cells) were infected with JFH-1 derived virions, and ARB was added 48 hrs or 24 hrs before infection, at the time of infection and 24 or 48 hrs post-infection. At 72 hrs post-infection the extracellular virus was collected for titration and cells were extracted for HCV proteins analysis by Western blot. Both assays gave concordant results and showed that ARB had the most pronounced effect in cells pre-treated for 48 or 24 hrs (up to 1000-fold reduction in virus yields), whereas reduced anti-viral effect were observed when ARB was added at the time of infection and minimal effects when ARB was added 24 or 48 hrs post-infection [23]. It should be emphasized that these studies are based on a single administration of ARB. In clinical scenarios, ARB is administered multiple times and over multiple days. Thus, additional studies are warranted to investigate potential therapeutic effects of ARB following multiple dosing. Nonetheless, the data demonstrate that a single dose of ARB is capable of blocking HCV infection in cell culture.

7.3. HCV Infection by HCV Pseudoparticles

ARB inhibited infection of Huh7 cells by HCV pseudoparticles in a dose-dependent manner, with 50% inhibition of infectivity at ARB concentration of 6 μg/ml. The maximum inhibitory effect was observed when cells were pre-incubated with ARB for 3 hrs before infection and left in the presence of ARB onwards, in which case only 1 μg/ml of ARB was sufficient to attain 50% inhibition of infectivity. Importantly, ARB inhibited infection by pseudoparticles bearing envelope proteins of HCV genotypes 1a, 1b and 2a, further attesting to the broad-spectrum properties of this antiviral compound [11].

7.4. HCV Inhibition by ARB is not Mediated by Interferon (IFN)

HCV replication is sensitive to IFN which directly inhibits HCV by multiple mechanisms [47]. Early studies on ARB suggested that besides its antiviral action the compound possessed immunomodulatory, anti-oxidant and IFN-inducing activities [5]. There is *in vitro* evidence for anti-oxidant activity of ARB and its derivatives based on their lipid radicals' scavenging in the course of lipid peroxidation of liposomes [48]. The observation in humans of a redistribution of T cell subsets after ARB administration [5, 36] suggested the immunomodulatory activity of ARB. ARB has also been suggested to induce IFN production since serum levels of IFN were higher in ARB-treated subjects [36].

We therefore investigated the ability of ARB to induce an IFN response as a possible cause of HCV inhibition using *in vitro* experiments. To this end, we determined the effects of ARB on innate antiviral signal transduction pathways in FL-Neo and Huh7 cells. We measured activation of the IFN-beta promoter by retinoic acid inducible gene 1 (RIG-1), a key factor in double stranded RNA signaling in response to HCV infection [49]. We also measured basal and IFN-alpha-induced IFN-stimulated response elements (ISRE) transcription, a measure of the activation of Jak-Stat (Janus kinases-signal transducers and activators of transcription) pathway activation *via* the ISGF-3 (IFN-stimulated gene factor 3) transcription factor, which is a complex of Stat 1 – Stat 2 – IRF9 (interferon regulatory factor 9) proteins [50]. ISRE activation occurs downstream of IFN-beta activation during virus infection [50, 51].

Transfection of FL-Neo replicon and Huh7 cells with RIG-N, a constitutively active mutant of RIG-1 [52], caused robust induction of IFN-beta transcription, as compared to cells that expressed control green fluorescent protein. Addition of ARB to cells did not modify the basal level of RIG-N-induced IFN-beta transcription. Rather, ARB caused a dose-dependent inhibition of IFN-beta transcription in all conditions. IFN-alpha treatment of FL-Neo and Huh7 cells activated ISRE transcription while ARB inhibited basal and IFN-induced ISRE promoter activity in a dose-dependent manner. Moreover, treatment of Huh7 and Huh7.5.1 cells with ARB did not induce phosphorylation on the conserved tyrosine amino acid at position 701 of the Stat1 protein, an essential requirement for and indicator of IFN signaling through the Jak-Stat pathway [52]. Finally, incubation of FL-Neo cells with ARB for up to 4 days did not increase the expression of IFN-stimulated genes (ISGs) Stat 1 and Stat 2. Collectively, the data indicate that ARB does not induce an IFN antiviral response in hepatocyte cultures that could account for the inhibition of HCV replication by ARB, and do not support the notion that ARB acts as an IFN inducer, at least in this *in vitro* cell culture system [23].

7.5. Mechanism of HCV Fusion Inhibition by ARB

We then examined the ability of ARB to block HCV at the viral entry level. Similarly to influenza virus, HCV enters target cells by endocytosis followed by a fusion step from within an acidic endosome compartment [53]. HCV-mediated fusion occurs within a broad pH range of 6.3 to 5.0, but is most efficient at pH 5.4 to 5.0 [54, 55]. For *in vitro* fusion experiments we used HCV pseudoparticles and liposomes mimicking endocytic vesicles. The liposomes contained a rhodamine-based fluorophore that emits fluorescence upon dilution of viral and liposome membranes, as a result of virus/liposome fusion. The lipid-mixing assay is quantitative and allows recording of fluorescence intensity as a measure of virus-mediated fusion [55].

At a pH optimum of 5.0, ARB caused inhibition of pseudoparticle fusion with liposomes in a dose-, time- and viral genotype – dependent manner. The most sensitive to ARB inhibition was genotype 1a (fusion was completely blocked at 1 μg/ml ARB concentra-

tion) followed by genotype 1b (6 μg/ml ARB concentration) and genotype 2a (over 6 μg/ml ARB concentration) [11].

ARB possesses intrinsic fluorescent properties with maximum excitation and emission wavelengths of 255 nm and 350 nm, respectively. This allowed us to study ARB interaction with detergent micelles as models of a membrane-like environment. At detergent concentrations well above critical micellar concentrations, the addition of ARB to micelles led to a dramatic increase in the quantum yields of fluorescence for three different detergents. This effect was more pronounced at pH 5.0 as compared to pH 7.4 that possibly reflects ARB transition from a deprotonated neutral state to a protonated cationic form. These data indicate that ARB has affinity for hydrophobic environments and is preferentially bound in micelles at acidic pH as a result of ARB altered ionization state.

Similar experiments were also performed in the presence of liposomes. As the concentration of phosphatidylcholine liposomes increased, the maximum emission wavelength of ARB progressively shifted to lower wavelength (blue shift) with a concomitant increase in quantum yield. That was an indication of a significant increase in hydrophobicity of ARB environment due to ARB interaction with lipid membranes. Even larger blue shifts (up to 20 nm) and greater increases in quantum yield were noted at pH 5.0 than at pH 7.4, suggesting enhanced incorporation of ARB molecules into the hydrophobic core of lipid bilayers at acidic pH. The extent of the blue shift was larger with liposomes than with detergent micelles. This may reflect rather superficial and loose ARB embedding in detergent micelles, in contrast to deeper hydrophobic penetration and tighter association in liposomal membranes [11].

Our results suggest at least two, although not mutually exclusive, assumptions regarding the pH-dependent mechanism of ARB inhibition of HCV fusion:

i. **ARB intercalates into lipids of the endosomal membrane, rendering it resistant to viral fusion**. Indole derivatives propensity to membrane interfaces is well known [8, 9, 10]. The S-phenyl group of ARB could also interact with the hydrophobic fatty acid chains of phospholipids inside the bilayer. The amino groups could bond the phosphate moieties of phospholipids and establish a salt bridge between two adjacent phospholipid molecules as an ion pair complex. At low pH these interactions would be favored due to the protonation of the amino groups. In particular, protonation of the 3-position could displace the ester group out of the indole plane and place it in a better position to bond with neighboring molecules. This could, in turn, lead to a better membrane association. Considering these chemically plausible interactions, ARB might have the propensity to intercalate into lipids in the membrane while adopting a consistent orientation by filling the gaps between lipid molecules. The molar ratio between ARB and lipids in our *in vitro* fusion assay is ca. 1:10, so a plausible mode of action could be the formation of a stable and dose-dependent "ARB cage" at the surface of membranes, therefore leading to excessive stabilization of these membranes, which become resistant to fusion [11].

ii. **ARB interacts with viral envelope proteins.** Fusion with liposomes induced by three HCV genotypes is inhibited by ARB to a different extent suggesting the role of genotype-specific factors. The only difference between HCV genotypes lies in their genetic and protein variations that involve sequence alterations and possibly conformation of E1/E2 proteins. In case of altered conformation of the E1/E2 heterodimer, the ARB molecule may dock in different positions on the genotype-specific viral fusion protein and exert various degree of fusion inhibition. This assumption can be tested when the E1/E2 3D structure becomes available.

Despite being a weak base, ARB has experimentally shown to have poor buffering capacity [11] and unlikely acts like other lysosomotropic agents by raising endosomal pH [56] to an extent that would be detrimental for HCV fusion with endosome membrane.

8. PERSPECTIVES

Despite the current availability of several antivirals for influenza, an ideal drug for efficient prophylaxis during flu outbreaks has yet to be developed. A recent meta-analysis [39] on the efficacy of currently available drugs concluded that amantadine or rimantadine use should be discouraged due to significant adverse events and swift onset of antiviral resistance. The neuraminidase inhibitors may cause viral resistance and are costly compared to adamantanes or ARB.

The available data show that ARB prophylactic and treatment efficiency is comparable to that of adamantanes or neuraminidase inhibitors whilst ARB seems to exceed the other two classes of drugs in terms of safety. The additional benefits of ARB in clinical use are the lack of selection of resistant viruses and its therapeutic effectiveness against influenza-like illnesses. That was proven in in-vitro studies against various respiratory viruses performed by several groups, and in a limited clinical trial that brought negative results on emergence of ARB-resistant influenza virus variants (26, Leneva, I.A., Burtseva, E.I, Shevchenko, E.I, Shuster, A.M. 46th Interscience Conference on Antimicrobial Agents and Chemotherapy. San Francisco, CA, September 27-30, 2006, p. 463). By its mechanism of action ARB is completely different from the M2 ion channel blockers (amantadine and rimantadine) or specific inhibitors of neuraminidase (zanamivir and oseltamivir) and, hence, would make a good candidate as an adjunct therapy to either of the above drug groups.

As outlined in [39], the viral load and virulence of pandemic viruses are considerably higher compared to seasonal influenza viruses. Therefore, it would seem logical to depart from influenza monotherapy to combination treatment with drugs targeting different viral functions in order to improve the efficacy and safety of influenza treatment. Indeed, experiments in cell culture have demonstrated synergistic effects of ARB with amantadine, rimantadine, ribavirin, ribamidil or neuraminidase inhibitors against influenza A and B viruses [5, 29].

Unlike the established medicinal use of ARB for influenza and respiratory illnesses' treatment, ARB application for hepatitis C has not yet been tested clinically. In the foreseeable future pegylated IFNs will likely remain the backbone of anti-HCV therapy with adjunctive use of small molecule compounds, such as ribavirin analogues, viramidine, levovirin or specific inhibitors of HCV enzymes [57].

A number of broad-spectrum marketed drugs originally designed for treatments against viruses other than HCV have demonstrated anti-HCV activity *in vitro*. The examples include sodium stibogluconate, an antimony–containing drug used in leishmania treatment [58], cyclosporine A, a potent immunosuppressive drug [59] and S-adenosylmethionine that is used for the treatment of alcoholic liver disease [60]. All of these drugs are licensed for use in human and have well documented toxicity and pharmacokinetic profiles that facilitate their new anti-HCV application. ARB joins the list of broad-spectrum anti-HCV agents but, in contrast to the incompletely defined viral or cellular targets of other agents, ARB has a deciphered mechanism of anti-HCV action [11]. ARB targets viral fusion, a common early step in the life cycle of many viruses. In the case of the influenza virus ARB affects the conformation of HA, critical for fusion due to a narrow pH-dependence of influenza virus-mediated fusion. Moreover, the pH optimum of membrane fusion and hemolysis mediated by antigenically distinct influenza viruses ranges from 5.0 to 6.0, depending on the virus strain. Previous observations [65,66] corroborated by our data indicate that a pH

increase of 0.2 units can significantly affect fusion as a result of conformational changes of HAs of wild type and drug-resistant viruses. In contrast, HCV fusion takes place within a much broader pH range, so the ARB effect on E1/E2 proteins could be secondary to ARB's propensity to interact with membrane to disrupt the membrane fusion activity of HCV.

At present, we do not know if ARB has effects on lipid rafts or caveolae, which are often involved in virus entry into cells [67]. We have shown that ARB interacts in a similar manner with liposomes that contain cholesterol [11], which is one component of lipid rafts. However, rafts also contain sphingomyelin, but we have not yet incorporated into liposomes. Clearly, additional studies are required to determine if ARB modulates these types of membrane microdomains.

ARB is a good example of how the same broad-spectrum drug targeting the same critical step in viral infection can affect either the viral or cellular counterpart in a virus - cell interaction depending on the viral context. An important point about ARB's anti-HCV action is that the drug not only prevents HCV acute infection but also cures chronic infection [23], at least *in vitro*. Chronic hepatitis C grossly outnumbers acute hepatitis C in terms of cases subject to treatment, since about 85% of acutely infected patients develop chronic infection [61].

Because ARB inhibited HCV replication in a replicon cell line that did not produce infectious particles, mechanisms other than blockade of HCV-endosome fusion must be invoked to explain these results. Since HCV replication occurs on intracellular membranous webs [63], and several HCV proteins have membrane binding functions that are required for virus replication [64], ARB may disrupt intracellular membrane functions that are required for replication, and thereby contribute to ARB's antiviral action.

As an administered drug, ARB has several workable ways for improvement in terms of solubility, therapeutic index (TI) and plasma concentration. In many of the *in vitro* experiments described above, ARB base was required to be initially dissolved in alcohol followed by adjustment with water, in order to achieve complete ARB dissolution and full antiviral activity. In some cases, ARB must be dissolved in other organic solvents such as DMSO. Clearly, synthesis of water-soluble ARB derivatives would facilitate the study of this interesting compound. In terms of TI, the most effective antivirals have TI's in the range of 100 – 1000, and this is particularly true for highly specific inhibitors of viral enzymes. The broad-spectrum and less specific antivirals inhibit virus replication indirectly and, generally, do not exhibit as high TI's. In our experiments, ARB TI's for influenza virus and HCV were around 5 and 1, respectively. Preliminary studies with ARB derivatives have increased the TI to 20, and some compounds are completely soluble in water (S.J. Polyak, unpublished).

In conclusion, ARB has proven to be an effective broad-spectrum antiviral as demonstrated in cell culture and animal studies. A vast clinical experience has accumulated on ARB prophylaxis and treatment of influenza and acute respiratory infections. Since its mechanism of antiviral action is different from other approved antivirals, ARB could be a good candidate as a pharmacologic enhancer when co-administered with other drugs. This would be particularly beneficial in the treatment of hepatitis C, by allowing the reduction of IFN dose while maintaining or improving the efficacy of treatment and dosing convenience.

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LIST OF ABBREVIATIONS

ARB	=	Arbidol
HCV	=	Hepatitis C virus
TI	=	Therapeutic index
MDCK	=	Madin-Darby canine kidney cells
HPLC	=	High-pressure liquid chromatography
MIC50	=	Minimal inhibitory concentration (50%)
CC50	=	50% cytotoxic concentration
IC50	=	50% virus-inhibiting concentration
EI	=	Efficacy index
IFN	=	Interferon
RIG-1	=	Retinoic acid inducible gene
ISRE	=	Interferon-stimulated response elements
Jak-Stat	=	Janus kinases – signal transducers
ISGF-3	=	Interferon-stimulated gene factor 3
IRF9	=	Interferon regulatory factor 9

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