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Characteristics of arbidol-resistant mutants of influenza virus: Implications for the mechanism of anti-influenza action of arbidol

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ABSTRACT

The antiviral drug arbidol (ARB), which is licensed in Russia for use against influenza, is known to inhibit early membrane fusion events in influenza A and B virus replication. To investigate in more detail the target and mechanism of ARB action we generated and studied the characteristics of ARB-resistant influenza virus mutants. Observations of the ARB susceptibility of reassortants between A/Singapore/1/57(H2N2) and A/chicken/Germany/27(H7N7, "Weybridge" strain) and of mutants of the latter virus identified the virus haemagglutinin (HA) as the major determinant of ARB sensitivity. ARB-resistant mutants, selected from the most sensitive reassortant, possessed single amino acid substitutions in the HA2 subunit which caused an increase in the pH of fusion and the associated conformational change in HA. ARB was shown to stabilize the HA by causing a 0.2 pH unit reduction in the pH of the transition to the low pH form, which was specifically abrogated by the resistance mutations. Some of the resistance mutations, which reduce acid stability and would disrupt ARB-HA interactions, are located in the vicinity of a potential ARB binding site identified using the docking programme *Gold*. Together, the results of these investigations indicate that ARB falls within a class of inhibitor which interacts with HA to stabilize it against the low pH transition to its fusogenic state and consequently inhibit HA-mediated membrane fusion during influenza virus infection.

1. Introduction

Influenza virus infection causes significant morbidity, mortality and economic loss worldwide. Prevention and treatment of influenza currently relies on vaccines and antiviral agents. Although vaccines are the better option for influenza control, their composition has to be updated regularly to reflect changes in the circulating viruses and at least 6 months are needed to produce new vaccines incorporating the most recent antigenic variants (Couch et al., 1996). In addition, some people are not adequately protected by vaccination (Ohmit et al., 1999; Powers and Belshe, 1993; Ruben, 1990). Consequently, the availability of several effective anti-influenza drugs is an important adjunct to vaccination. As with other viral infections, proteins that confer essential functions during the replication cycle are potential targets for the development of antiviral agents (De Clercq, 2006). To date only two have provided targets for anti-influenza drugs in widespread use.

Amantadine (1-aminoadamantane hydrochloride) and its derivative rimantadine (alpha-methyl-1-adamantane methylamine hydrochloride) are effective therapeutically and prophylactically against human infections by influenza A viruses, but not influenza B (Douglas, 1990; Hayden, 1996; Zlydnikov et al., 1981). They target the M2 proton channel of the virus that is necessary for virus uncoating to release the viral nucleocapsid prior to initiation of virus replication (reviewed in Hay, 1996).

Knowledge of the crystal structure of the virus neuraminidase(NA) permitted the design of two licensed drugs, inhaled zanamivir and orally effective oseltamivir (von Itzstein et al., 1993; Kim et al., 1997). They interrupt the latest stages in virus replication by preventing the release of progeny virions from infected cells, causing their aggregation and reducing virus spread to neighbouring cells. These anti-NA drugs are well tolerated and broadly effective in the prophylaxis and early treatment of both influenza A and B infections (Hayden et al., 1997, 1999; Nicholson et al., 2000; Sidwell et al., 1998).

Besides these two proteins, the other virus surface protein, the haemagglutinin (HA), is a particularly attractive target, especially

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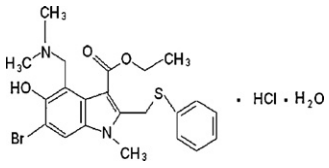

Fig. 1. The chemical structure of Arbidol.

since neutralizing antibody against HA is principally responsible for immunity to infection. The HA plays the key roles during virus entry in binding the virus to sialic acid receptors and in mediating fusion of virus and endosome membranes to initiate replication. The latter function occurs at the low pH of 5–6 within endosomes which triggers a conformational change in HA to its fusogenic form (Daniels et al., 1985; Skehel et al., 1982). Several studies have identified a class of compounds that inhibit viral infection by blocking the low pH-induced conformational changes of HA. Bodian et al. (1993) identified a group of benzo- and hydro-quinones that bind in a pocket of the H3 HA (but not H1 or H2) and prevent the native HA from undergoing the low pH-induced conformational change (Hoffman et al., 1997). In other reports a quinolizidine-linked benzamide was shown to block the HA conformational change of H1 and H2 (but not H3) subtypes (Luo et al., 1996, 1997). Plotch et al. (1999) also described several compounds that inhibit infectivity of H1, H2 (and to a lesser extent H3) subtypes by blocking the HA conformational change. Since the conformational change is key to the fusogenic activity of the HA and virus infection, this event presents a strategic target for pharmacological intervention (Meanwell and Krystal, 1996).

The antiviral drug arbidol (ARB) (1-metyl-2-phenyl-thiomethyl-3-carbotoxy-4-dimetylaminomethyl-5-hydroxy-6-bromo-indolehydrochloride monohydrate) (Fig. 1) was developed at the Russian Research Chemical and Pharmaceutical Institute and was shown to inhibit the replication of all subtypes tested of human influenza A and influenza B viruses in cell cultures. The IC_{50} values depended on the virus strain and ranged from approximately 3 to 9 μg/ml; for example for different type/subtype viruses they were 3.5, 7.5, 5.0, 9.0 and 5.0 μg/ml for A/Puerto Rico/8/34 (H1N1), A/Mississippi/85 (H2N2), A/Aichi/2/68 (H3N2), B/Lee/40 and B/Hong Kong/330/01, respectively (Leneva et al., 1994, 2005). ARB inhibited early stages of influenza virus infection; it was most effective if added before virus infection (Glushkov et al., 1992; Leneva et al., 1994) and it inhibited low pH-induced fusion between virus and the plasma membrane, as well as between virus and endocytic vesicle membranes (Glushkov et al., 1992; Fadeeva et al., 1992). These results therefore suggested that ARB also targets the HA of influenza virus.

ARB was shown to be effective in mice infected with A/PR/8/34 (H1N1) or A/Aichi/2/68 (H3N2) influenza A viruses. When administered orally 24 h before virus exposure and continued for 5 days, ARB at 60–120 mg/kg/day significantly reduced the virus titer in lungs as well as the mortality rate by 70–100% (Guskova and Glushkov, 1999; Shi et al., 2007). ARB displayed oral bioavailability of 38% in rats. Several randomized placebo-controlled clinical trials of ARB efficacy in the treatment and prophylaxis of influenza infection were conducted between 1983 and 1995 on more than 10,000 patients overall. In these trials ARB was well tolerated and no severe side effects were reported (Guskova and Glushkov, 1999). ARB administered orally at 200 mg daily, three times a day, for 5–10 days was shown to reduce the duration of illness by 1.7–2.6 days in patients infected with influenza, and to prevent the development of post-influenza complications and reduce the frequency of exacerbation of prior chronic respiratory diseases, such as chronic obstructive pulmonary disease, asthma, rhinitis and sinusitis. The therapeutic efficacy of ARB was most pronounced when the drug was administered early in infection. In several controlled trials in students and industrial workers, ARB prophylaxis was shown to be 80% effective during influenza A outbreaks in 1988–1989, with an optimum dose of 100 mg twice a day for 10–18 days. Daily oral administration of 200 mg doses of ARB for 3 weeks to patients in clusters of respiratory infections (e.g., families, hospital wards, etc.) during an epidemic of influenza B reduced the incidence of disease by 86% (Glushkov, 1992; Guskova and Glushkov, 1999; Shumilov et al., 2002, reviewed in Boriskin et al., 2008). Clinical trials have also shown efficacy of ARB in preventing and reducing influenza in children (Beliaev et al., 1996; Drinevskiy et al., 1998; Uchaikin et al., 2004). In 1990, ARB was approved for treatment and prophylaxis of influenza A and B infections and has since been widely used in Russia.

Numerous studies have demonstrated the utility of drug-resistant mutants in elucidating the mechanisms of action of anti-influenza drugs as well as the molecular basis of drug resistance (Daniels et al., 1985; Gubareva et al., 1996; Hay, 1996; Hoffman et al., 1997; Luo et al., 1996, 1997; Plotch et al., 1999; Tai et al., 1998). To better understand the mechanism of ARB action, we selected ARB-resistant mutants of influenza A virus, identified the drug-resistance mutations and investigated their effects on the activity of ARB in *in vitro* assays. In this paper we show that, like other inhibitors of fusion, ARB can interact with the HA to stabilize it against low pH-induced conformational changes which promote membrane fusion and conclude that this is the basis of its anti-influenza activity in cell culture.

2. Materials and methods

2.1. Cells and viruses

Madin Darby canine kidney (MDCK) cells were grown in minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS), 5 mM L-glutamine, 25 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 100 μg/ml kanamycin sulfate, in a humidified atmosphere of 5% CO_2. The A/Singapore/1/57 (H2N2) and A/chicken/Germany/27 (H7N7) "Weybridge" viruses were from the World Influenza Centre stock at NIMR, Mill Hill, London. The reassortants between A/Singapore/1/57 and A/chicken/Germany/27 "Weybridge" viruses (19b, 9a, 7a, 21b, 43a, 58a), amantadine-resistant (C5, G12, A2, A5, C13) and norakin-resistant (N37) A/chicken/Germany/27 "Weybridge" mutants used in this study were described previously (Daniels et al., 1985; Grambas and Hay, 1992).

2.2. Preparation of ARB stock

ARB free-base powder was dissolved to completion in 0.5 ml of 96-proof ethanol at 37 °C for 10 min followed by dilution in 4.5 ml of sterile distilled water. For each experiment a freshly made stock was used. This stock was used for preparation of required ARB concentrations in all experiments.

2.3. Selection of ARB-resistant mutants

The 7a reassortant, which was the most sensitive to ARB, was chosen for selection of ARB-resistant mutants. MDCK cells grown in 24-well plates, were infected with different dilutions of allantoic virus at 0.1–0.01 PFU/cell. Virus growth in the presence or absence of ARB was determined at each passage by the hemagglutination (HA) assay. Virus from the highest dilution which still gave a HA titre, was used for each subsequent passage. Virus did not grow initially in the presence of 10–20 μg/ml ARB; thus, its

concentration was increased gradually from 5 to 20 μg/ml during successive virus passages using 5, 7.5, 10, 12.5, 15, 17.5 and 20 μg/ml ARB. By the 6th passage, the virus appeared to have adapted to 10 μg/ml ARB in MDCK cells (mutant M1), and by the 14th passage to 20 μg/ml of the drug (mutants 201, 202, 203, 204, 206). ARB-resistant mutant F1 was isolated following six viral passages in chick embryo fibroblasts in the presence of 10 μg/ml of ARB. ARB-resistant viruses were plaque purified in MDCK cells, grown in 6-well tissue culture plates under agar overlay containing ARB (20 μg/ml), with the exception of the F1 mutant which was plaque-purified in CEF. Viral plaques were picked using sterile 200-μl pipette tips, resuspended in 1 ml PBS, and virus stocks were grown in the allantoic cavities of 11-day-old embryonated hen eggs for use in subsequent experiments. The allantoic 7a virus was passed in MDCK cells in exactly the same manner as the ARB-resistant mutants, but without ARB, for 14 passages and served as a control virus.

2.4. HA titration

HA titration was performed using 50 μl of the supernatants from various passages, with addition of 50 μl of PBS and 50 μl of 0.5% chicken erythrocytes.

2.5. Virus plaque assay

Confluent MDCK cell monolayers in 6-well tissue culture plates were incubated with ARB at concentrations of 5, 10 or 20 μg/ml in MEM for 30 min at 37 °C. ARB was removed and after washing with medium, cells were inoculated with virus diluted in PBS to give the inoculum from 50 to 100 PFU per well. The cells were left for 1 h at room temperature to allow virus to adsorb, the virus inoculum was removed and cells were overlaid with double-strength 199 medium containing 0.75% Noble Agar, 1% FCS, 0.2% BSA and 0.001 DEAE-dextran, with or without ARB. Plaques were visualized in agar overlaid with neutral red on the third day after infection.

2.6. Assaying antiviral activity of ARB by ELISA

A modified enzyme-linked immunoassay (ELISA) (Belshe et al., 1988; Grambas and Hay, 1992) was used to measure the inhibition of virus replication by ARB. This assay detected the expression of viral HA 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 °C with 5% CO_2 until 90% cell confluency was reached, then washed twice with serum-free MEM before infection. Each microtiter plate included uninfected control wells, virus-infected control wells and virus-infected wells to which ARB was added. Cells were overlaid with MEM (100 μl) containing 2.5 μg/ml *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin and various ARB concentrations. Viruses (reassortants and mutants) containing the HA from A/chicken/Germany/27 "Weybridge" strain did not require addition of trypsin. After incubation for 30 min at 37 °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 °C in a humidified atmosphere of 5% CO_2 cells were washed and fixed by adding 50 μl of cold 0.05% glutaraldehyde in PBS. Expression of HA protein was measured by ELISA as described previously (Belshe et al., 1988; Sugrue et al., 1990). Antibodies HC58 (10^{-3} dilution), for viruses that had A/chicken/Germany/27 "Weybridge" HA, and JapHC18 (2×10^{-3} dilution), for viruses that had HA from A/Singapore/1/57 strain, were used. The percentage inhibition of virus replication by ARB was calculated after correction for the background (cell control) values as follows: Percent inhibition = $100 \times [1 - (OD_{450})$ 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.

2.7. Study of the pH-induced HA conformational changes by ELISA

MDCK cells in 96-well plates were prepared as described above and infected with A/chicken/Germany/27 "Weybridge" viruses (m.o.i. approximately 100 PFU per cell). After 5.5 h incubation at 37 °C in serum-free MEM, cells were washed, and incubated at 37 °C for 15 min in a series of PBS-citrate buffers (50 μl per well). The pH of the buffers ranged from 4.6 to 7.4, with 0.2 pH unit increments (15 different pHs). After 15 min cells were immediately fixed by adding 50 μl of cold 0.05% glutaraldehyde in PBS, and the HA expression was measured by ELISA. Ascitic fluids containing antibodies to either the native form of HA (HC58, 10^{-3} dilution) or the low-pH form of HA (H9, 10^{-2} dilution) of the A/chicken/Germany/27 "Weybridge" virus, were used.

To investigate the effect of ARB on the HA conformational changes, the above procedure was carried out with the following modifications. After 5.5 h incubation at 37 °C in serum-free MEM medium, cells were washed and treated by the appropriate concentrations of ARB at 37 °C for 30 min. ARB was then withdrawn, cells were washed and incubated at 37 °C for 15 min with 50 μl PBS-citrate buffer at different pH within the range of 4.6–7.4, as above. After fixation of cells with 50 μl of cold 0.05% glutaraldehyde in PBS, the ELISA was carried out as described above. Each point represented the mean of four ELISA readings (HA expression in four replicate wells) in the same experiment.

2.8. Haemolysis assay

Virus-induced haemolysis was estimated as described previously (Wharton et al., 1994). All virus preparations had equal HA activity. The wild-type virus 7a or mutant viruses were adsorbed to 50% human erythrocytes suspension, and incubated at different pH from 7.4 to 4.8 at 37 °C for 10 min. The amount of haemoglobin released into the cell supernatant was estimated spectrophotometrically at 540 nm. The ΔpH of ARB-resistant viruses *vs*. wild-type virus was determined based on the pH value at which 50% haemolysis occurred. To investigate the effect of ARB on haemolysis, ARB concentrations of 50, 75, 100 or 200 μg/ml were added to virus-erythrocyte suspensions at pH 5.0.

2.9. Nucleotide sequence analyses

Viral RNA was extracted from samples of allantoic fluid by phenol-chloroform followed by ethanol precipitation. Two μl of RNA was reverse-transcribed and amplified by use of a one-tube RT-PCR kit (Boehringer Manheim) according to the manufacturer's instructions. The HA-specific primer pairs were H7A1F1 (5′-AGCAAAAAGGAGGGGTTACAAAATG-3′, nucleotides 1-25) and H7A2F1 (5′-CCACGATATGTAAAACAGGAAAGT-3′, nucleotides 964-987), H7A1F1 and H7A1R1 (5′-TATGGCGCCAAACAGGCCTCTTTT-3′, nucleotides 1042-1065), and H7A2F1 and H7A2R1 (5′-CCAAACTTATATACAAATAGTGCA-3′, nucleotides 1696 -1719). The PCR products were purified using MicroSpin™S-400 HR columns (Amersham Biosciences) and sequenced using the ABI Prism dye terminator cycle sequencing kit and an ABI Model 377 DNA Sequencer (PerkinElmer, Applied Biosystems). The sequencing primers were H7A1F1, H7A1F2 (5′-GGAAATGATGTTTGTTACCC-

Table 1
Genome composition and ARB-sensitivity of reassortants between A/Singapore/1/57 and A/chicken/Germany/27 (Weybridge strain) viruses.

Virus	IC_{50} (μg/ml)	Gene[a]							
		1	2	3	4	5	6	7	8
		PB2	PB1	PA	HA	NP	NA	M	NS
A/ck/Germany/27	4.5	W	W	W	W	W	W	W	W
7a	3.0	W	W	W	W	W	W	A	W
19b	5.0	W	A	A	W	A	W	W	A
21b	5.0	W	W	A	W	W	A	A	W
9a	5.0	A	A	A	W	A	A	A	W
43a	10	W	W	W	A	W	W	W	W
58a	12	A	A	A	A	A	A	A	W
A/Singapore/1/57	10	A	A	A	A	A	A	A	A

[a] W and A represent genes from A/chicken/Germany/27 (Weybridge strain) and A/Singapore/1/57, respectively.

GGGG-3′, nucleotides 322-345), H7A1R2 (5′-TCGTGTTCCTGGA-CTCGGCACAAA-3′, nucleotides 685-707) and H7A1R1 for HA1, and primers H7A2F1 and H7A2R1 for HA2. For the M gene, sequencing primers Eco R1 TAGGGAATTCCAAAAGCAGG and Bgl II CAAGAAGATCTTTTACTAAAG-CTCTAT, were used.

3. Results

3.1. Effect of ARB on the replication of different reassortants and drug-resistant viruses

To investigate which virus gene(s) influence the susceptibility to ARB, we studied a series of reassortants between A/Singapore/1/57(H2N2) and A/chicken/Germany/27(H7N7; "Weybridge" strain) viruses, which differ in sensitivity to ARB. The genetic compositions of the reassortants are listed in Table 1. They fall into two groups based on their sensitivity to ARB (Fig. 2A) and origin of their HA gene. One group, including reassortants 7a, 9a, 19b and 21b that possess the Weybridge HA, exhibited high sensitivity to ARB; their replication was inhibited by greater than 80% with 8 μg/ml ARB, IC_{50} 3–5 μg/ml. The second group of reassortants that included 43a and 58a, was inhibited by less than 50% with 10 μg/ml ARB, IC_{50} 10–12 μg/ml, and possessed the HA of A/Singapore/1/57. There was no correlation between ARB sensitivity and the presence of any gene other than HA, in these reassortants.

We also examined the ARB susceptibility of a number of mutants of the same Weybridge H7 strain that were previously selected for resistance to high concentrations of other anti-influenza drugs, including amantadine (A5, G12, C5 and C13), norakin (N37) and biperiden (Bip11 and Bip14) since they had mutations in the HA affecting the pH of fusion (Daniels et al., 1985; Grambas and Hay, 1992). ARB inhibited replication of some of the amantadine-resistant mutants, A5 and G12, with an IC_{50} of 7–8 μg/ml, somewhat higher than for the Weybridge HA-containing reassortants, whereas replication of the amantadine-resistant mutant C13, the norakin-resistant mutant N37 and the biperiden-resistant mutants, Bip 11 and 14 was resistant to ARB, and inhibited by 25% or less at 20 μg/ml, a concentration which completely inhibited the former (Fig. 2B). These results suggest some similarity between the action of ARB and those of high concentrations of amantadine, norakin and biperiden, and are consistent with the results of the reassortant experiments which indicate that the HA is the major determinant of ARB susceptibility.

3.2. ARB-resistant mutants

Reassortant 7a, the most sensitive of the reassortants, was used to select ARB-resistant mutants following passage with increasing concentrations of the drug, up to 20 μg/ml (see Section 2.3). The mutants M1, F1, 201, 202, 203, 204 and 206 were tested for sensitivity to ARB in the MDCK cell ELISA. All were relatively resistant to ARB to 20 μg/ml (IC_{50} > 20 μg/ml), as shown for mutants 202, 204 and M1 in Fig. 3. In addition, a plaque reduction assay showed that mutant M1 was refractory to 10 μg/ml ARB, whereas the wild-type 7a virus was completely inhibited (data not shown).

Nucleotide sequences obtained for the HA and M genes of the wild-type 7a and ARB-resistant viruses showed that all mutants had mutations in the HA gene which caused single amino acid substitutions at four different positions in the HA2 subunit (Table 2). Mutants 201, 203, 204 and 206, selected with 20 μg/ml ARB, had the same mutation, K51N, mutant 202 had a Q42H mutation, mutant

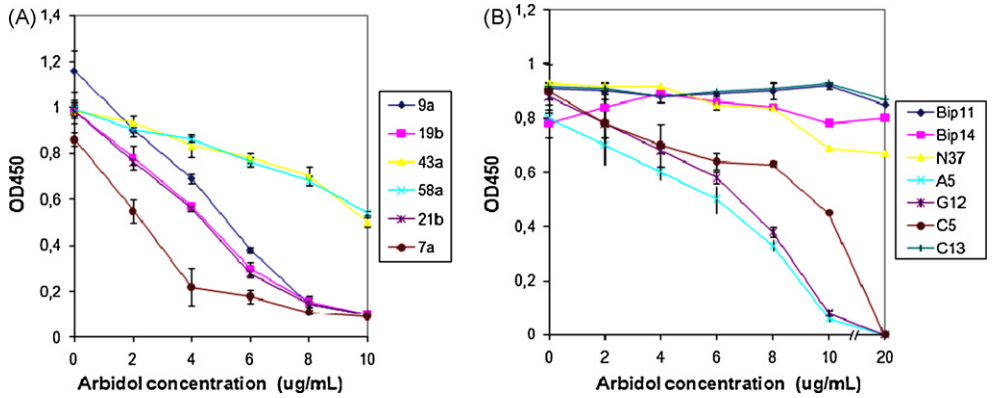

Fig. 2. Effect of ARB on the replication of (A) reassortants between A/Singapore/1/57 and A/chicken/Germany/27 viruses, (B) drug-resistant mutants of A/chicken/Germany/27. MDCK cells were grown in 96-well plates and infected with viruses in the presence of different concentrations of ARB. The levels of HA protein expression were measured by ELISA, as described in Section 2. Each point represents the mean of four replicate wells in one experiment.

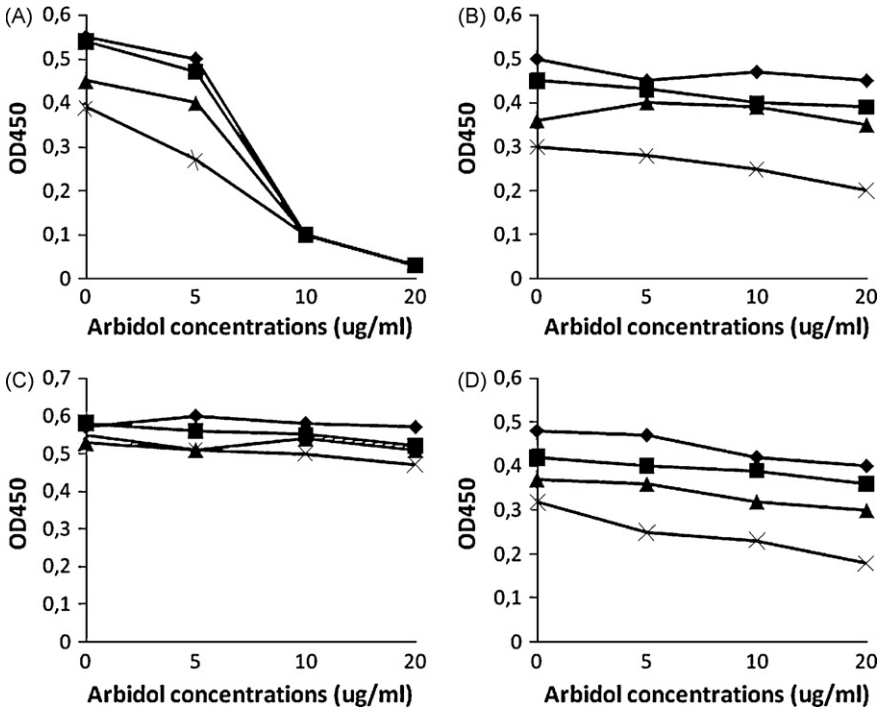

Fig. 3. Drug susceptibility of ARB-resistant mutants. MDCK cells in 96-well plates were infected with 7a wild-type virus (A) or ARB-resistant mutants 202 (B), 204 (C) or M1 (D) at MOI of 20 (♦), 10 (■), 5 (▲) or 1 (**X**) PFU/cell. The levels of HA expression were measured by cell ELISA, as described in Section 2. Each point represents the mean of HA expression in four replicate wells in one experiment.

M1 had a Q27N mutation and mutant F1 had a K117R mutation. The nucleotide sequences of the M genes revealed no substitutions in either M1 or M2 proteins of the ARB-resistant mutants. The sequence of the 7a control virus that was passed in MDCK cells without ARB revealed only single nucleotide changes which, unlike ARB-resistant mutant sequences, did not resulted in amino acid substitutions. A single Q42H mutations was also present in the HA of resistant mutant N37, selected by passage in the presence of Norakin, whereas Bip11 and Bip 14 had the single changes D112G and T107A, respectively.

3.3. Inhibition of virus-induced haemolysis by ARB

Our previous studies using a fluorescence dequenching assay indicated that ARB inhibition occurred at an early stage of influenza virus infection of cells by inhibition of virus–cell membrane fusion at pH 5.0 (Glushkov et al., 1992; Fadeeva et al., 1992). We, therefore, compared the effect of ARB on red blood cell lysis induced by wild-type virus or ARB-resistant mutants at pH 5.5, the approximate pH of haemolysis of 7a. The results showed that ARB inhibited haemolysis induced by the wild-type virus maximally, by approximately 80%, at 100 μg/ml. In contrast, ARB concentrations as high as 200 μg/ml did not affect haemolysis induced by ARB -resistant mutants M1 or 204 at pH 5.5.

3.4. pH-dependence of membrane fusion by wild-type and mutant viruses

The pH-dependence of the haemolytic activities of the ARB-resistant mutants were compared with that of wild-type 7a virus over the pH range 7.4–4.6. The differences in pH at which haemolysis was reduced by 50% (ΔpH) are listed in Table 2, and an example of the difference in pH dependence of haemolysis by the wild-type virus and one of the mutants (M1) is given in Fig. 4. Fifty percent haemolysis occurred at pH ~5.6 for the wild-type virus, and at ~5.8 for mutant M1. Differences were approximately 0.2 or 0.4 pH units higher for the other ARB-resistant mutants (Table 2).

During low pH-induced fusion the influenza virus HA undergoes an irreversible conformational change that can be specifically detected by differences in reactivity with conformation-specific monoclonal antibodies (Grambas et al., 1992; Sugrue et al., 1990). We assayed the pH of the transition in HA conformation of wild-type and ARB-resistant mutants by ELISA using antibody HC58, that specifically recognizes native HA, and antibody H9, that specifically

Table 2
Amino acid substitutions in ARB-resistant mutants and consequent increases in the pH of fusion.

ARB-resistant mutant	Residue	Amino acid substitution	Increase in pH of fusion	
			ΔpH of transition[a]	ΔpH of haemolysis[b]
M1	HA2 27	Q→N	0.2	0.4
202	HA2 42	Q→H	0.2	0.2
201, 203, 204, 206	HA2 51	K→N	0.2	0.2
F1	HA2 117	K→R	0.2	0.2

[a] The pH of the conformational transition was measured by ELISA using conformation-specific antibodies, as described in Section 2. The shift in pH (ΔpH) of transition for each mutant, relative to wild-type, did not differ significantly in three separate experiments.

[b] The ΔpH of haemolysis was determined from the pH dependence of haemolysis for each mutant compared with wild-type virus (Fig. 4), as described in Section 2. Results did not differ significantly in three separate experiments.

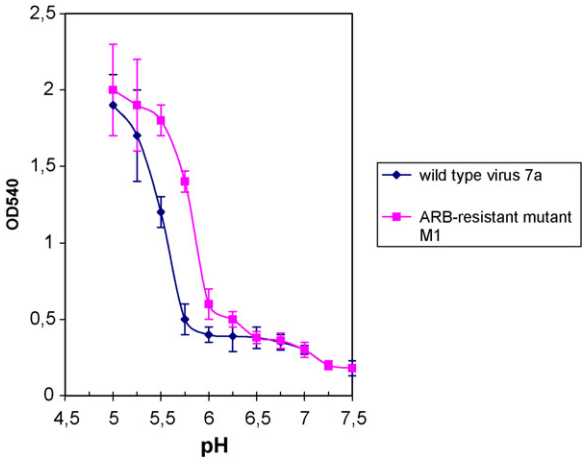

Fig. 4. pH-dependence of haemolysis by 7a and ARB-resistant mutant M1. Assays were performed using equal HA titres of virus, as described in Section 2.

recognizes the HA in its low pH conformation. For the wild-type HA the conformational transition occurred at pH ~5.0, whereas for the HA of ARB-resistant mutants it occurred at pH ~5.2 (Table 2, Fig. 5). The results of both experiments demonstrate that the ARB resistance mutations cause an increase of approximately 0.2 units in the pH required to induce the conformational change in HA and consequent membrane fusion.

3.5. *Effect of ARB on the conformational change in HA of wild-type and mutant viruses*

Although the data indicated that ARB inhibits fusion activity of the virus HA *in vitro*, it was not clear whether ARB interacts directly with the HA or inhibits the fusion process indirectly, e.g., by elevating the pH in endocytic vesicles of infected cells. We attempted to resolve this issue by investigating the effect of ARB on HA produced in cells following a single cycle of infection by the wild-type or ARB-resistant viruses. After 5.5 h infection with the wild-type 7a or ARB-resistant mutants 201 or 202, cells were treated with 10–50 μg/ml ARB for 30 min and then for 15 min with different pH from 7.4 to 4.6, before analysing the HA conformation using

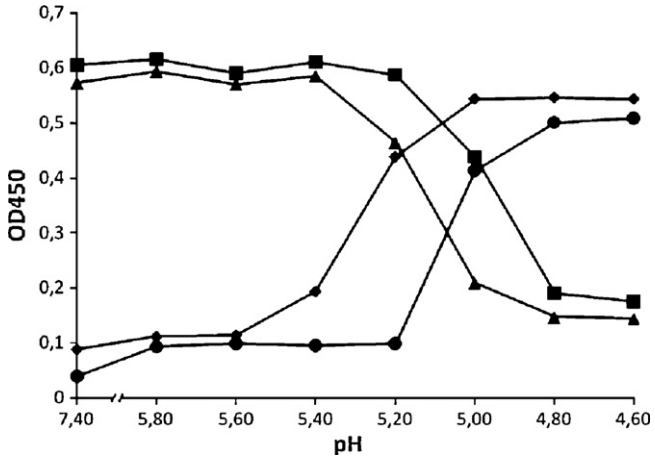

Fig. 5. pH dependence of the conformational change in HAs of mutant 202 and wild-type 7a. 5.5 h after infection with mutant (▲,♦) or wild-type 7a (■,●) viruses, cells in 96-well plates were incubated at 37 °C for 10 min with PBS-citrate at different pH, ranging from 4.6 to 7.4. Cells were fixed with 0.05% glutaraldehyde and HA was detected by ELISA using antibodies HC58 (■,▲) or H9 (●,♦). Each point represents the mean of four replicate wells in one experiment.

conformation-specific antibodies (Section 2.7). For wild-type HA, the addition of ARB, 10–50 μg/ml, caused a 0.2 unit decrease in the pH of the conformational transition from 5.0 to 4.8. In contrast, ARB had no effect on the pH of the conformational change (pH 5.2) of HA on cells infected by mutants 201(K51N) or 202(Q42H) (Fig. 6). Furthermore, studies of amantadine-resistant mutants also showed a correlation between inhibition of virus replication by ARB (Fig. 2B) and an ARB-induced reduction in the pH of the conformational change in HA. The K51N mutation in HA2 abolished sensitivity of the HA of C13 to an ARB-induced 0.2–0.3 pH unit reduction in the conformational transition of the HAs of the three ARB-sensitive viruses, A5, C5 and G12 (data not shown). The increase in the pH of the conformational transition of the latter three mutant HAs, 0.1–0.4 pH units relative to wild-type (Daniels et al., 1985), and the amino acid substitutions responsible, R91L and R91Q in HA1 of A5 and G12, respectively, and F3L in HA2 of C5, did not counteract the stabilizing effect of ARB. Together these results indicate, therefore, that ARB interacts with HA to increase its stability to low pH-induced conformational change and consequently inhibit membrane fusion in endosomes during virus infection, and that ARB resistance mutations interfere specifically with this interaction.

4. Discussion

Our previous studies showed that ARB did not affect virus binding and attributed the mechanism of its anti-influenza action to inhibition of fusion between virus and endosomal membranes (Glushkov et al., 1992). The results of the present studies have extended these findings by identifying HA as a target of ARB action and showing that ARB can interact directly with HA to stabilize it against low pH-induced conformational change and prevent HA-mediated membrane fusion.

The CC_{50} (50% cytotoxic concentration) of ARB was shown to be 40–60 μg/ml (Fediakina et al., 2005; Leneva et al., 1994) and the therapeutic indices (SI) of ARB in cells for most viruses ranged from 3 to 10. However, cytotoxicity in cell culture may not reflect the toxicity of the drug in animals and humans. Clinical trials conducted in more than 10,000 patients and experience of using ARB over 15 years in Russia have shown that ARB is well tolerated and no side effects have been revealed.

Similar concentrations of ARB were required for the inhibition of virus growth in cell culture (IC_{50} ~10 μg/ml) and for reducing the pH of the low pH-induced conformational changes in HA. However, a fivefold higher concentration of ARB was required for inhibiton of haemolysis. The exact reason for this discrepancy is not clear but could be associated with differences in virus interaction with red blood cell *vs.* mammalian tissue culture cell membranes.

ARB resistance mutations, which map to the HA2 subunit, caused increases in the pH of the conformational change and membrane fusion, and specifically abrogated the stabilizing effect of ARB. It is apparent therefore that ARB does not act indirectly like high concentrations of amantadine and other acidotropic amines and prevent infection by elevating the pH of endosomes, which is then counteracted by reduced acid stability of drug-resistant mutants, but interacts directly with the HA and that the resistance mutations interfere with this interaction or its consequences. The action of ARB is therefore similar to that of the variety of small molecules which comprise the class of fusion inhibitors that disrupt the structural rearrangements which render HA fusogenic (Bodian et al., 1993; Luo et al., 1997; Hoffman et al., 1997; Cianci et al., 1999; Plotch et al., 1999). However, their specificity for different influenza A subtypes varies, limiting their potential usefulness, as does the spectrum of amino acid substitutions which cause resistance, suggesting that the different compounds inter-

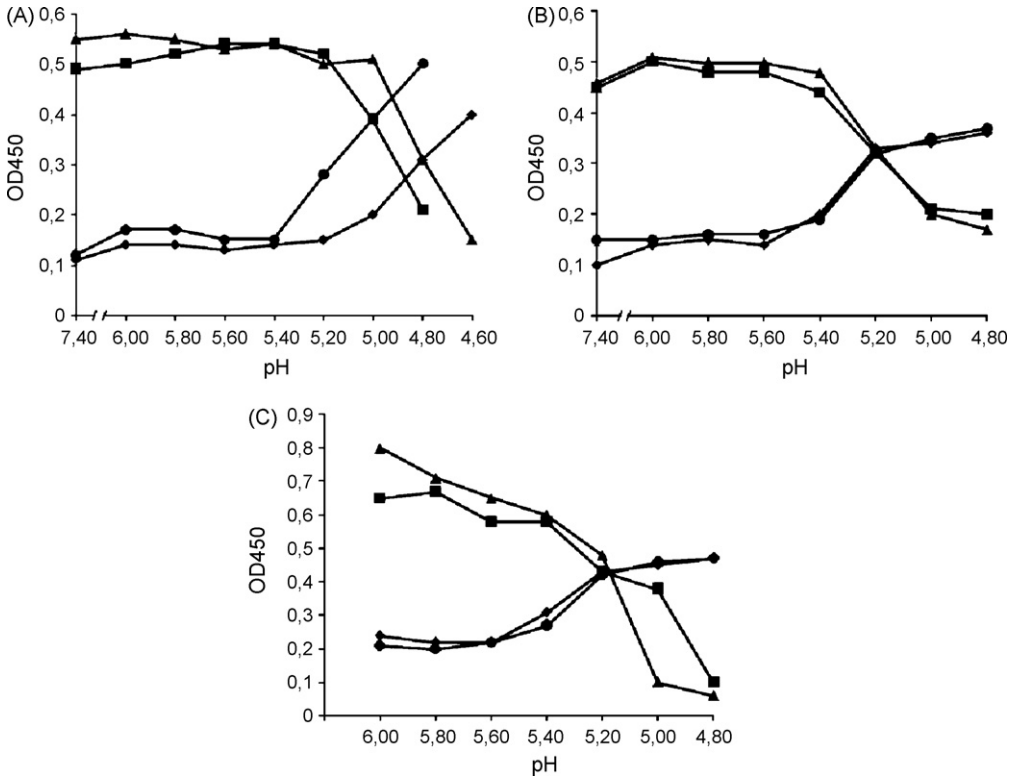

Fig. 6. The effect of ARB on the HA conformational change of wild-type 7a virus (A) and the ARB-resistant mutants 201(B) and 202(C). 5.5 h after infection with wild-type 7a or mutants 201 or 202, cells were washed and untreated (■,●) or treated (▲,♦) with ARB (50 μg/ml) at 37 °C for 30 min. ARB was then withdrawn and cells were washed and incubated at 37 °C for 15 min with 50 μl PBS-citrate buffer at different pH within the range 4.6–7.4. Cells were fixed with 0.05% glutaraldehyde and HA was analysed by ELISA using antibodies specific for either native HA, HC58 (■,▲) or the low pH, H9 (●,♦). Each point represents the mean of HA expression in four replicate wells in one experiment.

act in different ways and possibly at different sites on the HA molecule.

A docking programme *Gold* was used to identify a cavity close to the N-terminus of the fusion peptide of HA2 into which ARB could fit (Fig. 7). Although this shows that a cavity of a size suitable to accommodate ARB does exist in the H7 HA, it is too speculative to provide any bases for considering the mechanism of interaction of ARB with HA or the influence of ARB resistance mutations, especially when only one of the four mutations is in the vicinity. The destabilizing effects of two ARB resistance mutations (K51N and K117R) are, however, apparent. Lys51 makes hydrogen bonds to both His106 and Thr107 and the introduction of Asn at this position would eliminate these stabilizing interactions, resulting in an increase in the pH of acid-induced changes. Substitution of Thr107 by Ala, in the resistant mutant Bip14, removes the hydrogen bond to Lys51 and thus eliminates a stabilizing interaction. Lys117 forms hydrogen bonds to the side-chain of Glu114 and to the main chain carbonyl of Gly1 of an adjacent HA2 subunit; mutation to Arg would cause steric clashes to both these interactions and necessitate altered conformations which eliminate the stabilising interactions. For the other two ARB resistance mutations, Q27N and Q42H, there is, however, no obvious explanation for their destabilizing effects. The Asp112Gly mutation in Bip11 removes hydrogen bonds from the side chain to the fusion peptide main chain amide groups.

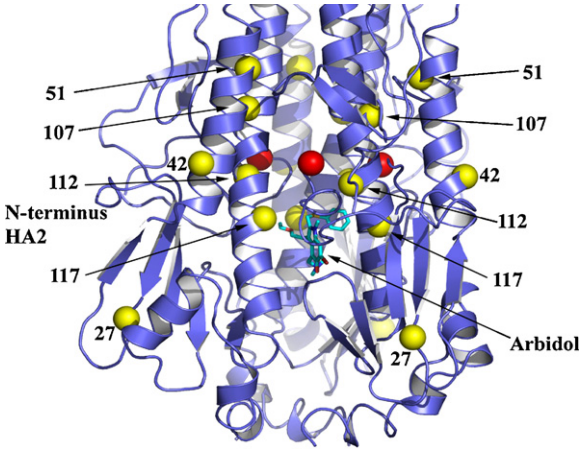

Fig. 7. Diagram showing the locations of ARB resistance mutations in relation to a cavity, large enough to accommodate ARB, near the N-terminal fusion peptide of HA2 of the H7 trimer (Russell et al., 2004). The cavity was identified using the docking program *Gold*. The N-termini of HA2 are displayed as red spheres and the locations of the ARB resistance mutations (including those selected in the presence of amantadine, norakin or biperiden) as yellow spheres. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Mutations in some of these positions were selected for resistance to some other inhibitors with a similar mechanism of action. For example, substitution of Lys117 by Glu (K117E) in HA2 of X-31 abrogated inhibition by *tert*-butyl hydroquinone (TBHQ) (Hoffman et al., 1997). K51R, together with Y119H, in HA2 of WSN (H1N1) was selected for resistance to BMY-27709 (4-amino-5-chloro-2-hydroxy-*N*-9-αH-octahydro-6β-methyl-2H-quinolizin-2α-benzamide) (Luo et al., 1997). Photoaffinity radiolabelling by an azido derivative of an analogue, BMS-201160, was used to identify a location in HA2, close to the fusion peptide, to which the inhibitor bound (Cianci et al., 1999). Given the complexity of the low pH-induced structural rearrangements in HA, an explanation of the precise nature of the binding interactions and the mechanisms of inhibition and resistance will require detailed structural data. Attempts to soak ARB into a crys-

tal of H7 of A/turkey/Italy/2002 proved unsuccessful and further attempts will require the Weybridge HA.

Although we have demonstrated clearly that *in vitro* ARB can interact with HA to disrupt the low pH-induced conformational changes necessary for its fusion activity during virus infection, the variation in sensitivity of different HAs (and subtypes) raises questions as to the basis of its clinical efficacy. In this respect, of the different actions of high and low concentrations of amantadine against HA and the M2 proton channel, respectively, the frequent selection of amantadine resistance mutations in M2 *in vivo* has identified the specific inhibition of the M2 channel as the clinically effective action. No such data are available for ARB.

Selection of ARB-resistant mutants by serial passages in the presence of increasing concentrations of ARB required considerably more effort than selection of amantadine- and rimantadine-resistant mutants, 15 passages for ARB *vs*. only 2–3 passages for amantadine- and rimantadine-resistant mutants (reviewed in Hay, 1996). This may account for the fact that whilst ARB has been in clinical use in Russia for many years, ARB-resistant viruses have yet to be identified (Burtseva et al., 2007). In this context, the frequency of detection of viruses resistant to the neuraminidase inhibitors, zanamivir and oseltamivir, was relatively low prior to the recent emergence of oseltamivir-resistant H1N1 viruses (Lackenby et al., 2008), despite extensive surveillance. Furthermore, although NA gene sequences were not determined in this study, mutations in NA or some other virus component(s) may be required to compensate for adverse effects of mutations in HA to preserve the fitness of resistant viruses.

The outbreak of influenza A(H5N1) in Hong Kong in 1997 and its persistence since 2003 and spread to three continents is a reminder that the threat of a new deadly influenza pandemic is ever present (De Jong et al., 1997; Abdel-Ghafar et al., 2008). Our *in vitro* experiments have shown that ARB inhibits the replication of recent isolates of avian influenza H5N1 and H9N2 subtype viruses in MDCK cells with IC_{50}s of 4–9 μg/ml, within the range required for inhibition of human influenza viruses (Fediakina et al., 2005; Lvov et al., 2006). In addition, ARB is orally administered and broadly distributed within the body, which is important in cases of possible systemic disease such as H5N1 infection (De Jong et al., 2005). Given the increasing instances of the development of resistance to the anti-M2 and anti-NA drugs, it is important to identify the basis of the clinical efficacy of ARB so that it might enhance the armoury of drugs more generally available to combat influenza and more specifically as a first line defence against a pandemic.

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