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Molecular Mechanism Underlying the Action of a Novel Fusion Inhibitor of Influenza A Virus

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In the initial stages of influenza virus infection, the hemagglutinin (HA) protein of influenza virus mediates both adsorption and penetration of the virus into the host cell. Recently, we identified and characterized BMY-27709 as an inhibitor of the H1 and H2 subtypes of influenza A virus that specifically inhibits the HA function necessary for virus-cell membrane fusion (G.-X. Luo, R. Colonno, and M. Krystal, Virology 226:66–76, 1996). Studies presented herein show that the inhibition is mediated through specific interaction with the HA protein. This binding represses the low-pH-induced conformational change of the HA protein which is a prerequisite for membrane fusion. In an attempt to define the binding pocket within the HA molecule, a number of drug-resistant viruses have been isolated and characterized. Sequence analyses of the HA gene of these drug-resistant viruses mapped amino acid changes responsible for drug resistance to a region located near the amino terminus of HA2. In addition, we have identified inactive analogs of BMY-27709 which are able to compete out the inhibitory activity of BMY-27709. This finding suggests that inhibition of the HA-mediated membrane fusion by this class of compounds is not solely the result of binding within the HA molecule but requires specific interactions.

Membrane fusion is believed to be a ubiquitous process used by enveloped viruses to transfer their genetic material into host cells (46, 47). In most cases, fusion events between viral and cellular membranes are mediated by virus-specific envelope proteins. In general, these viral proteins contain a stretch of hydrophobic amino acids within the extracellular domain that promotes the process of membrane fusion (46, 47). The hemagglutinin (HA) protein of influenza virus is one of the best-characterized viral proteins which can induce membrane fusion (17, 46–48).

The HA protein of influenza virus is a glycoprotein anchored in the viral membrane and is present as a homotrimer of three molecules (30, 48). Each HA monomer is expressed as a single polypeptide precursor (HA0), which is proteolytically cleaved into two polypeptide subunits, HA1 and HA2, covalently linked by a single disulfide bond (30, 48, 49). The atomic structure of the ectodomain of the H3 subtype HA, solubilized from the viral membrane by bromelain digestion (BHA), has been determined (49). Structurally, each HA molecule consists of two major domains. One is a large distal globular head domain which is composed of the HA1 subunit and contains a sialic acid binding pocket along with the major antigenic sites on the molecule. The other domain contains a fibrous stem formed mainly by α-helical structures from the HA2 subunit. A striking feature of the BHA structure is that the hydrophobic amino terminus of HA2, the so-called fusion peptide, is buried in the interior of the HA trimer, about 100 Å from the top of the globular head and 35 Å from the viral membrane (49). The native HA molecule mediates the binding of virus to the sialic acid-containing receptor protein on the host cell. However, the native HA is unable to mediate the membrane fusion event which occurs in the endosome prior to uncoating (14–17, 19, 38, 42–48). After binding to receptors, influenza virus gets internalized and traverses the endosomal pathway, where it becomes exposed to an increasingly acidic pH. The acidic pH triggers a conformational change, such that the native form of HA trimer undergoes an irreversible structural rearrangement which renders it fusion competent. A key feature of the so-called fusogenic HA is the release of the hydrophobic fusion peptide from the interior of the trimeric HA (11, 17, 34, 43, 45–48). The exposed fusion peptide is believed to be inserted into the cellular membrane, where, through a yet undefined mechanism, it triggers the fusion of the viral and endosomal membranes (4, 12, 15, 21, 29, 39, 46–48). Evidence that this hydrophobic peptide plays a major role in promoting fusion has been obtained from systems using either synthetic peptide or the solubilized ectodomain of the HA protein (4, 29, 33, 37). In addition, it was recently demonstrated that the fusion peptide of the HA2 ectodomain is the only region which becomes radiolabeled when target membranes are spiked with specific photoaffinity ligands, suggesting that this region inserts into the target membrane before fusion (12).

A number of theories have been proposed in an attempt to model the conformational changes which occur in the HA in order to expose the fusion peptide and insert it into the lipid bilayer (36, 47). A key finding was made by Carr and Kim (6); through the use of synthetic peptides and molecular modeling, they suggested that the loop sequence between two α-helices within the stem of the HA2 can refold into an α-helix upon exposure to low pH. This structural feature has been confirmed by recent X-ray crystallographic studies of proteolytic fragments of the HA (5, 19). This finding allowed for a model

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whereby the loop-to-helix switch exposes the fusion peptide and projects it 100 Å upward toward the target membrane (6).

The unique mechanism of membrane fusion induced by the HA molecule makes it a potential target for antiviral intervention (6, 7, 17, 48). Bodian et al. (3) used a computer-assisted docking program to identify small molecules which could bind in a pocket of the H3 HA and possibly prevent the native HA from undergoing the low-pH induced conformational change. From this analysis, a number of benzoquinone- and hydroquinone-containing compounds which can inhibit the low-pH-induced conformational change of the X31 (H3N2) virus HA were identified. We recently identified and characterized a specific inhibitor of influenza A virus which is active against virus growth in tissue culture (24). This compound, BMY-27709, specifically inhibits the growth of both H1 and H2 subtypes of influenza A virus but is inactive against H3 subtype viruses. Through the use of reassortant, drug-resistant, and transfectant viruses, it was demonstrated that the target of BMY-27709 is the HA protein. Furthermore, in vitro virus-mediated hemolysis experiments have demonstrated that BMY-27709 is a novel fusion inhibitor of influenza A virus (24). The data presented here show that the basis for inhibition of the HA-mediated membrane fusion by BMY-27709 is through blockage of the low-pH-induced conformational change of HA. Based on the amino acid mutations of drug-resistant viruses and through examination of the three-dimensional structure of the H3 HA, molecular modeling techniques identified a potential binding pocket for BMY-27709. In addition, it was discovered that certain close analogs of BMY-27709 which are not inhibitory for HA-induced membrane fusion can actually antagonize the activity of BMY-27709. These antagonists are proposed to bind in the same pocket of the HA as BMY-27709 but lack the specific interaction(s) necessary for inhibition of the low-pH-induced conformational change of HA.

MATERIALS AND METHODS

Cells and viruses. Madin-Darby bovine kidney (MDBK) cells were grown in minimal essential medium (MEM; GIBCO, Gaithersburg, Md.) with 10% fetal bovine serum (FBS; Sigma, St. Louis, Mo.). The virus-infected MDBK cells were maintained in MEM containing 0.42% bovine albumin solution (Miles, Kankakee, Ill.). CV-1 cells were grown in MEM with 10% FBS. Influenza A/WSN/33 (H1N1) virus and drug-resistant variants were grown in MDBK cells. Influenza A/PR/8 (H1N1) virus was propagated in 11-day-old embryonated hen eggs (24). Chicken erythrocytes (RBCs) were purchased from Spafas (Preston, Conn.).

Hemolysis assay. The hemolysis inhibition assay to examine reversibility of the effect of BMY-27709 was modified from that described previously (24). Briefly, influenza A/WSN/33 virus was incubated without (control) or with 37 μM (greater than the 90% inhibitory concentration) BMY-27709 at 37°C for 1 h. Then 200 μl of 2% chicken RBCs was added, and the mixture was incubated at 37°C for 10 min. Subsequently, the reaction was diluted up to various levels (3-, 9-, 27-, and 81-fold) by the addition of 1× cold phosphate-buffered saline (PBS). In an additional control, the virus was continually treated with BMY-27709 at 37, 12.3, 4.1, 1.37, or 0.46 μM, which is equivalent to the concentration attained after 0-, 3-, 9-, 27-, or 81-fold dilution. After incubation on ice for 5 min, the virus-bound RBCs were pelleted by centrifugation at 1,600 rpm for 8 min. The RBC pellet was resuspended in 450 μl of low-pH buffer (pH 5.0 ± 0.05) without drug and incubated at 37°C for 15 min to allow for hemolysis. The reaction was then neutralized to pH 7.0 ± 0.2 by the addition of 1 N NaOH. Cell debris and unlysed cells were pelleted by centrifugation at 2,000 rpm for 8 min. Then 300 μl of supernatant was transferred to a 96-well tissue culture plate (Corning Glass Works, Corning, N.Y.) for measurement of optical density (OD) at a wavelength of 540 nm, using a programmed Multiscan MCC/340 (Titertek) plate reader.

The pH at which the wild-type and BMY-27709-resistant viruses induced the lysis of chicken RBCs was determined by measuring the amount of hemoglobulin released at a wavelength of 540 nm following incubation at a given pH as described previously (24). The ΔpHs of resistant viruses compared to wild-type virus were determined based on the pH which caused 50% hemolysis.

Assay for antagonists of hemolysis inhibition. Influenza A/WSN/33 virus was incubated with 18.9 μM BMY-27709 (agonist) and various concentrations of the antagonists BMS-198254 (17.6, 70.2, 140.5, 280.9, 561.9, and 1,123.8 μM) and BMS-195161 (1.2, 4.7, 18.8, 75.3, and 301.3 μM) at 37°C for 1 h. Hemolysis assays were then run as described above and in reference 24. The activity of antagonist was measured by its ability to reverse the inhibition of hemolysis by BMY-27709. As controls, BMS-198254 and BMS-195161 were incubated either with RBCs alone or with influenza A/WSN/33 virus in the absence of BMY-27709. To examine if BMY-27709 can compete out prior binding of antagonists BMS-195161 and BMS-198254, A/WSN/33 virus was first incubated with either 301.3 μM BMS-195161 or 561.9 μM BMS-198254 at 37°C for 1 h. BMY-27709 was then added at a concentration of 18.5, 37, 74, or 148 μM, with an additional 30-min incubation at 37°C. The hemolysis was then assayed as described above.

ELISA detection of the HA conformational change. Immulon II flat-bottom plates (Dynatech Laboratories, Chantilly, Va.) were used for an enzyme-linked immunosorbent assay (ELISA) to detect native and low-pH forms of the A/PR/8/34 virus HA protein. Monoclonal antibodies against either the native form (H28) or low-pH-induced form (Y8) of HA of influenza A/PR/8 virus were generously provided by J. Yewdell (National Institutes of Health, Bethesda, Md.). Five micrograms of influenza A/PR/8 virus was added to each well of the 96-well assay plate and allowed to absorb at room temperature for 2 h. After one wash with 1× PBS, the plate was blocked by incubation with 1× PBS containing 3% bovine serum albumin at room temperature for 2 h. The virus-coated plate was washed twice with PBS, 50 μl of PBS containing various concentrations of BMY-27709 was added to each well, and the plates were incubated at room temperature for 15 min. The solution was removed, and the wells were treated with 50 μl of either pH 7.0 or pH 5.0 PBS containing various concentrations of BMY-27709 at room temperature for 15 min. Solution was removed, and wells were washed once with 1× PBS. Fifty microliters of a 3% bovine serum albumin solution containing either the H28 or Y8 monoclonal antibody was then added to each well, and plates were incubated at room temperature for 30 min. An alkaline phosphatase-conjugated secondary antibody (goat anti-mouse immunoglobulin G) was used to determine the amount of monoclonal antibody present (45, 50).

Trypsin susceptibility assay. HA protein used in this assay was expressed from virus-infected CV-1 cells. CV-1 cells in 35-mm^2 dishes were infected with either influenza A/WSN/33 virus or a BMY-27709-resistant virus at a multiplicity of infection of 10. At 1 h postinfection, 2 ml of MEM was added to each dish. At 6 h postinfection, the cells were washed twice with 1× PBS (GIBCO) and labeled with 1 ml of methionine-depleted MEM (GIBCO) containing [^{35}S]methionine (100 μCi/ml; NEN, Boston, Mass.) at 37°C for 2 h. After a 30-min chase with MEM, the cells were lysed in 500 μl of radioimmunoprecipitation assay buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholic acid, 0.1% sodium dodecyl sulfate [SDS]). Insoluble materials were pelleted by centrifugation at 12,000 × *g* for 15 min at 4°C. The supernatant was recovered and diluted with an equal volume of buffer B (0.1 M Tris [pH 7.6], 0.5 M NaCl). To assay the trypsin sensitivity of the HA protein, 38 μl of cell lysate was incubated with 2 μl of various concentrations of BMY-27709 at 31°C for 15 min. The pH of the reaction was then lowered to 5.0 ± 0.1 by the addition of a predetermined amount of 0.25 M citrate buffer (pH 4.2). After 15 min of incubation at 31°C, the reaction was neutralized to pH 7.0 ± 0.2 by the addition of 0.25 M Tris-HCl buffer (pH 9.0). The reaction mixture was subsequently incubated with 200 μg of trypsin (Sigma) at 37°C for 1 h. The reaction was terminated by the addition of 10 μl of soybean trypsin inhibitor (10 mg/ml; Sigma) for 20 min at 37°C and then immunoprecipitated by incubation with H17, a monoclonal antibody against H1 subtype HA (50, 51) (kindly provided by J. Yewdell). The immunoprecipitation was accomplished by overnight incubation with H17 at 4°C, followed by the addition of 50 μl of protein G-Sepharose (Sigma) at room temperature for 1.5 h. The pelleted products were analyzed on an SDS–12% polyacrylamide gel (22) and visualized with a STORM phosphorimager (20).

Isolation of resistant virus. Influenza A/WSN/33 virus was initially double plaque purified in MDBK cells. Twenty independent plaques were each used to infect one 35-mm^2 dish of MDBK cells. At 1 h postinfection, the virus-infected MDBK cells were incubated with 2 ml of REM medium (24) in the presence of 75.8 μM BMY-27709. After 3 days of incubation, supernatant from each dish was used to plaque virus in MDBK cells in the presence of 75.8 μM BMY-27709. Resistant virus was double plaque purified in the presence of compound, and each virus stock was amplified in MDBK cells in the presence of 75.8 μM BMY-27709.

Extraction of viral RNA; HA cDNA cloning and sequencing. Each BMY-27709-resistant virus was purified through 30 to 60% sucrose gradients. Virion RNA was extracted as described previously (23). The HA cDNA was amplified by reverse transcription and PCR using oligonucleotide primers and cloned into pUC19 as described previously (24). Two independent cDNA clones for each BMY-27709-resistant virus were sequenced by the dideoxynucleotide chain termination method (31). Mutations found in HA cDNAs were verified by direct RNA sequencing using commercial procedures (U.S. Biochemical, Cleveland, Ohio).

RESULTS

Reversibility of inhibition by BMY-27709. BMY-27709 was previously identified as an HA-specific inhibitor of H1 and H2

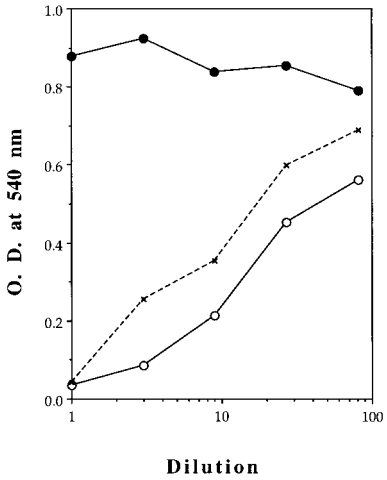


FIG. 1. Reversibility of hemolysis inhibition. Influenza A/WSN/33 virus was incubated with 37 μM BMY-27709 at 37°C for 1 h, followed by the addition of chicken RBCs. After BMY-27709-treated viruses were bound to RBCs, the reaction was diluted 3-, 9-, 27-, and 81-fold by the addition of PBS. The virus-bound RBCs were then pelleted and incubated in a low-pH (5.0) buffer at 37°C for 15 min prior to neutralization. Alternatively, hemolysis was assayed in the continued presence of BMY-27709 at a concentration of 37, 12.3, 4.1, 1.37, or 0.46 μM, which is equivalent to the concentration attained after 0-, 3-, 9-, 27-, or 81-fold dilution (dashed curve). The OD is plotted against the number of dilutions (fold) in log scale. ●, effect of dilution on hemolysis when BMY-27709 was not present; ○, reversibility of BMY-27709 inhibition following dilution.

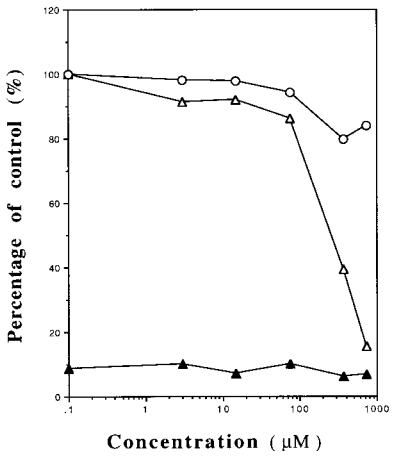


FIG. 2. Inhibition of HA conformational change by BMY-27709. Influenza A/PR/8 virus bound to an ELISA plate was preincubated with 0, 3, 14.8, 74, 370, and 740 μM BMY-27709 in a neutral pH buffer at room temperature for 15 min. The preincubation buffer was then replaced with citrate-buffered saline (pH 5.0) containing the same concentrations of BMY-27709. After 15 min of incubation at room temperature, the low-pH buffer was removed and the wells were washed with PBS. For mock treatment controls, all steps indicated above were done with a neutral-pH buffer. The confirmation of the bound HA was then determined by measuring the differential binding of monoclonal antibodies Y8 (acid form) and H28 (all forms [50, 51]). The percentage of inhibition is expressed as percentage of OD reduction compared to that of the reaction without BMY-27709 (100%). The percentage of inhibition is plotted against concentration. ○, H28 antibody; △, Y8 antibody at pH 5.0; ▲, Y8 antibody at pH 7.0.

subtypes of influenza A virus in tissue culture. Subsequent experiments showed that BMY-27709 was able to inhibit virus-mediated hemolysis of RBCs in vitro (24). Since the low-pH-induced conformational change of HA is essential for triggering the fusion process (11, 14, 17, 44, 47, 48), it was logical to assume that BMY-27709 may function by affecting this mechanism. However, BMY-27709 may act either by prematurely promoting the conformational change or through inhibition of the low-pH-induced conformational change of HA. Either mechanism will result in the same outcome: inactivation of the fusion activity of the HA protein. However, if BMY-27709 promotes the conformational change of HA, then the inhibition observed in an HA-mediated hemolysis assay would be irreversible. To examine this, virus was incubated with BMY-27709 at 37°C for 1 h prior to the addition of chicken RBCs. The reaction was subsequently diluted to decrease the concentration of BMY-27709, the RBCs were pelleted, and a hemolysis assay was performed. The results (Fig. 1) clearly show that the inhibition of hemolysis by BMY-27709 is reversible, as BMY-27709 activity correlated inversely with increasing dilution. As an additional control, the hemolysis assay was performed in the continued presence of BMY-27709 at concentrations of 37, 12.3, 4.1, 1.37, and 0.46 μM, which are equivalent to those attained after 0-, 3-, 9-, 27-, and 81-fold dilution. Reduction of hemolysis inhibition by BMY-27709 is proportional to the decrease of the concentration and very similar to what is measured in a dilution experiment (Fig. 1). This finding was also reproduced by an experiment in which, rather than simple dilution and decreasing the concentration, BMY-27709 was washed out with 1× PBS after BMY-27709-treated viruses were bound to RBCs (data not shown). These results demonstrate that inhibition of hemolysis by BMY-27709 is through noncovalent binding of BMY-27709 to the HA protein.

Inhibition of the HA conformational change by BMY-27709. To provide direct evidence for inhibition of the low-pH-induced conformational change of HA, an ELISA was used to monitor the appearance of the low-pH form of the HA in the presence of BMY-27709 (45, 50). Influenza A/PR/8/34 virus was incubated with BMY-27709 and treated with either neutral-pH (as control) or low-pH (pH 5.0) buffer. After neutralization of the low-pH-treated virus, the fusogenic form of HA

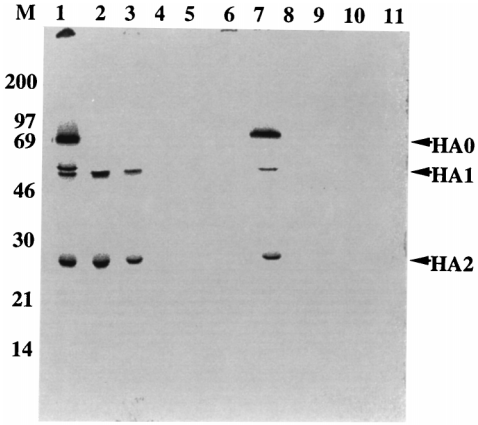


FIG. 3. Inhibition of the HA susceptibility to tryptic digestion by BMY-27709. CV-1 cells infected with either wild-type influenza A/WSN/33 (lanes 1 to 6) or a BMY-27709-resistant virus (lanes 7 to 11) were labeled with [^{35}S]methionine and lysed with radioimmunoprecipitation assay buffer. The lysate supernatants were incubated at 31°C for 15 min either in the presence of BMY-27709 at 740 μM (lanes 2 and 8), 74 μM (lanes 3 and 9), 7.4 μM (lanes 4 and 10), and 0.74 μM (lane 5) or in the absence of BMY-27709 (controls); (lanes 6 and 11). The pH of the reaction mixture was lowered to 5.0 ± 0.1, and then the mixture was neutralized and digested with trypsin. Lanes 1 and 7 are controls without low-pH treatment and trypsin digestion. The HA protein products were immunoprecipitated with monoclonal antibody H17 (50), analyzed by electrophoresis on a 12.5% polyacrylamide–SDS gel (22), and visualized with a STORM phosphorimager (20). Positions of size markers (lane M) are indicated in kilodaltons.

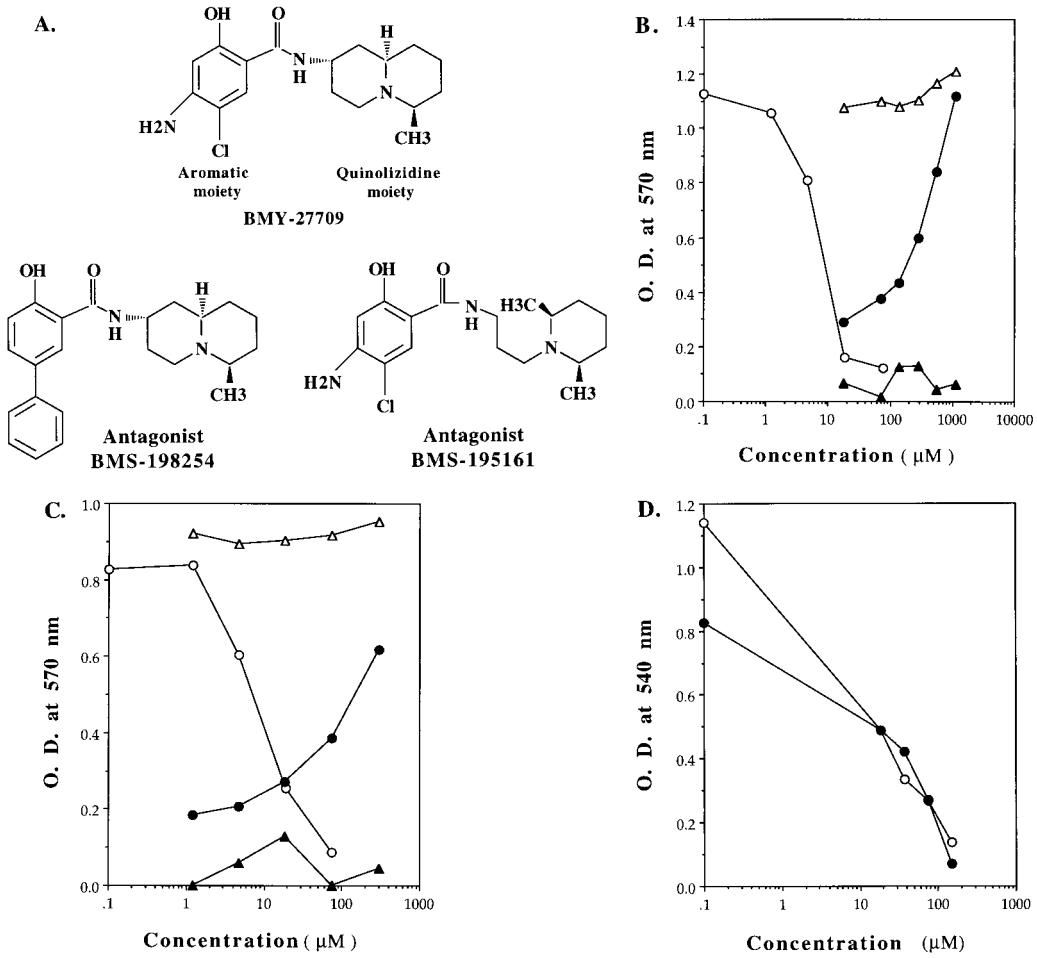


FIG. 4. (A) Structures of BMS-198254 and BMS-195161. (B) Antagonism of BMY-27709 activity by BMS-198254. Influenza A/WSN/33 virus was incubated with either BMY-27709 (○) at a concentration of 0, 1.2, 4.7, 18.9, or 75.8 μM or with 18.9 μM BMY-27709 in the presence of 17.6, 70.2, 140.5, 280.9, 561.9, or 1,123.8 μM antagonist BMS-198254 (●). In addition, A/WSN/33 virus was incubated with the above-mentioned concentrations of BMS-198254 alone (△). After incubation of virus and compounds, RBCs were added for the hemolysis assay. ▲, a control in which RBCs were incubated with BMS-198254 at the above-mentioned concentrations in the absence of virus. The OD is plotted against compound concentration (log scale). (C) Antagonism of BMY-27709 activity by BMS-195161. Influenza A/WSN/33 virus was first incubated with either BMY-27709 alone (○), 18.9 μM BMY-27709 and the antagonist BMS-195161 (●) at a concentration of 1.2, 4.7, 18.8, 75.3, or 301.3 μM, or BMS-195161 alone at the above-mentioned concentrations (△). As an additional control, BMS-195161 was incubated with RBCs only (▲). (D) Binding of antagonists BMS-195161 and BMS-198254 to HA is reversible. A/WSN/33 virus was first incubated with either 301.3 μM BMS-195161 (●) or 561.9 μM BMS-198254 (○) at 37°C for 1 h and then with BMY-27709 at a concentration of 0, 18.5, 37, 74, or 148 μM. The subsequent procedures were the same as described above. The OD is plotted against the concentrations (log scale) of BMY-27709.

was detected by using a monoclonal antibody (Y8) specific for the low-pH HA form of influenza A/PR/8 virus (50, 51). As shown in Fig. 2, BMY-27709 specifically inhibited the low-pH-induced conformational change of the HA protein, as measured by the reduction of the Y8 antibody reactivity with increasing concentrations of BMY-27709. In control experiments, the Y8 antibody did not react with the native HA which was treated with BMY-27709 at neutral pH (Fig. 2). Also, use of an antibody which reacts with either the native or the low-pH form (H28 [50]) shows that the decrease in reactivity with Y8 is not the result of loss of protein from the plates or the inhibition of antigen-antibody reactivity by BMY-27709. This experiment directly demonstrates that BMY-27709 inhibits fusion by preventing the native HA from undergoing the low-pH-induced conformational change.

Inhibition of the HA susceptibility to tryptic digestion by BMY-27709. The native trimeric HA protein is assembled in the viral membrane in such a way that it is resistant to trypsin digestion. Once exposed to a low pH, the HA trimer refolds and becomes susceptible to trypsin (34). To further confirm that BMY-27709 blocks the low-pH-induced conformational change, a tryptic digestion of HA was examined in the presence of BMY-27709. In addition, this assay was used to measure the trypsin susceptibility of an HA protein from a resistant A/WSN/33 virus (24). Radiolabeled CV-1 cell extracts infected with either wild-type virus or a resistant virus with a Phe-to-Ser change in the HA2 subunit were treated with BMY-27709 and then adjusted to low pH (5.0). After neutralization, extracts were digested with trypsin and immunoprecipitated with a monoclonal antibody against the HA protein (50). As shown in Fig. 3, the trypsin susceptibility of the wild-type HA protein increased as the concentration of BMY-27709 was decreased, so that by 7.4 μM, no HA protein was protected from trypsin digestion. However, the HA protein of a drug-resistant virus remained sensitive to trypsin digestion even at BMY-27709 concentrations up to 740 μM. This result further confirms that BMY-27709 prevents the HA trimer from undergoing the low-pH-induced conformational change and that this drug-resis-

TABLE 1. Amino acid mutations, drug sensitivity, and pH changes in hemolysis of BMY-27709-resistant viruses

Subunit	Amino acid substitution(s)	No. of viruses isolated	Resistance level[a]	ΔpH[b]
HA2	Asn50→Ser	1	15–25	0.1
	Lys51→Arg; Tyr119→His	1	>100	0.2
	Val52→Met	1	>100	0.1
	Val55→Ala	1	>100	0.2
	Phe110→Ser[c]	4	>100	0.3
	Phe110→Leu	1	15–25	0.1
	Ser166→Leu	1	>100	−0.1
HA1	Tyr7→His	1	10	0.0
	Ile19→Val	2	>100	0.0
	Met313→Val	7	>100	0.0
	Met313→Ile	1	>100	0.0

[a] Estimated fold EC_{50} increase versus wild-type virus based on the reduction of number of plaques.
[b] The pH at which 50% of hemolysis of the wild-type virus is 5.65.
[c] Includes virus described in reference 24.

tant HA is not affected by BMY-27709 treatment. It should be noted that variable amounts of the uncleaved HA0 protein are usually present in cell lysates and that this form of HA is susceptible to trypsin cleavage even in the presence of BMY-27709 (Fig. 3, lanes 2 and 3). Presumably, this HA0 is made up mostly of immature protein in the process of being formed into trimers and transported to the cell surface. This protein would therefore exhibit trypsin susceptibility in either the presence or absence of BMY-27709.

Antagonists of the inhibitory activity of BMY-27709. It was observed that subtle chemical modifications of BMY-27709 were able to inactivate its inhibitory activity against influenza virus. Specific chemical modification on either the aromatic or the quinolizidine heterocycle (Fig. 4A) resulted in inactive compounds (Fig. 4B and C). This finding suggests that both of these moieties are required for activity. However, it is not known whether the inactivity of these analogs is simply due to the loss of binding of these compounds to the HA protein. It is possible that these compounds can still bind to the HA protein but do not engage in some specific interactions that are required to prevent the HA from undergoing the low-pH-induced conformational change. This concept was tested in an altered RBC hemolysis assay. A constant inhibitory concentration of BMY-27709 (18.9 μM) was added with increasing concentrations of BMS-198254 and BMS-195161. If the inactive compounds can bind to the same pocket of the HA as BMY-27709, they may be able to antagonize the inhibitory activity of BMY-27709. The results from these experiments are shown in Fig. 4B and C. Interestingly, both inactive compounds (BMS-198254 and BMS-195161) are able to antagonize the activity of BMY-27709, as increasing concentrations allow for greater RBC hemolysis. Significant excesses of the antagonists are needed to compete out the activity of BMY-27709, suggesting that they may have lower binding affinities (Fig. 4B and C). BMS-198254 (Fig. 4B) was able to completely reverse the inhibition of BMY-27709, while BMS-195161 could not completely reverse the inhibition, possibly because of its poorer solubility (Fig. 4C). However, it appears that BMS-195161 is more potent than BMS-198254 in antagonizing the activity of BMY-27709 (Fig. 4B and C). This could be due to the fact that the bulk aromatic group attached to the phenyl ring moiety of BMY-27709 may affect the binding, since a derivative of BMY-27709 without the amino group (NH_2) in the phenyl ring moiety is more potent than the parent compound (unpublished data). In any case, these results suggest that binding to the HA is by itself not sufficient for inhibition and that specific interactions of both the aromatic moiety and the quinolizidine heterocycle of BMY-27709 with HA protein must occur in order to obtain inhibitory activity.

As described above, BMS-195161 and BMS-198254 can antagonize the inhibitory activity of BMY-27709 in a hemolysis assay, suggesting that they may bind in the same pocket of the HA protein as BMY-27709. Alternatively, it is also possible that these antagonists do not have the same binding sites as BMY-27709 but instead destabilize or distort the neutral pH conformation of HA through binding to another portion of the HA. To exclude this possibility, influenza A/WSN/33 virus was first incubated with either 301.3 μM BMS-195161 or 561.9 μM BMS-198254 at 37°C for 1 h and then incubated with increasing concentrations of BMY-27709. As shown in Fig. 4D, BMY-27709 can inhibit hemolysis in a concentration-dependent manner even with prior incubation of HA with the antagonists BMS-195161 and BMS-198254. This experiment further suggests that the antagonistic activities of BMS-195161 and BMS-198254 are not due to distortion of the neutral pH conformation of HA.

Genetic analysis of BMY-27709-resistant viruses. As suggested by the experiments described above, the ability of BMY-27709 to inhibit fusion appears to be dependent on its specific interactions with the HA protein. To better understand how BMY-27709 binds to the HA protein, efforts were made to select multiple drug-resistant viruses. Previously, we had described a single resistant virus with a phenylalanine-to-serine change at amino acid 110 of the HA2 subunit, which caused a resistant phenotype (24). Twenty additional independent resistant viruses were selected through propagation of viruses in the presence of 75.8 μM BMY-27709. The resistant viruses were then isolated by plaquing in MDBK cells in the presence of the compound and amplified, and virion RNAs were extracted from purified virions. Subsequently, cDNAs of the HA genes of the resistant viruses were constructed (24), and two independent HA cDNA clones of each resistant virus were sequenced. Nucleotide differences from the wild-type sequence which result in amino acid changes were verified by direct RNA sequencing. All 21 viruses contained mutations in their HA genes. The amino acid substitutions from these resistant viruses are shown in Table 1. Most of the resistant viruses have a single amino acid mutation in the HA protein (Table 1). Only one virus contains multiple mutations in the HA (Lys51 to Arg and Tyr119 to His, both in the HA2 subunit). All 21 viruses are resistant to BMY-27709 inhibition in a hemolysis assay (data not shown), which suggests that these changes are responsible for drug resistance in tissue culture. There are a total of 12 different mutations in both the HA1 and HA2 subunits. Interestingly, there are two major hot spots accounting for more than 50% of the resistant viruses (Table 1). One is the methionine at position 313 of the HA1 subunit, which mutated to either a valine (seven viruses) or an isoleucine (one virus). The other is phenylalanine at position 110 of the HA2 subunit, which is mutated to either a serine (four viruses) or a leucine (one virus). If H1 HA adopts a three-dimensional structure similar to that of the H3 HA, most of the amino acid substitutions would be located in the same region in the molecule. This region is located slightly above the N terminus of the HA2 subunit, which encodes the hydrophobic peptide believed to play a pivotal role in membrane fusion (Fig. 5).

Phenotypes of the resistant viruses. Resistant viruses were tested for sensitivity to BMY-27709 by plaquing in MDBK cells in the presence of various concentrations of the compound.

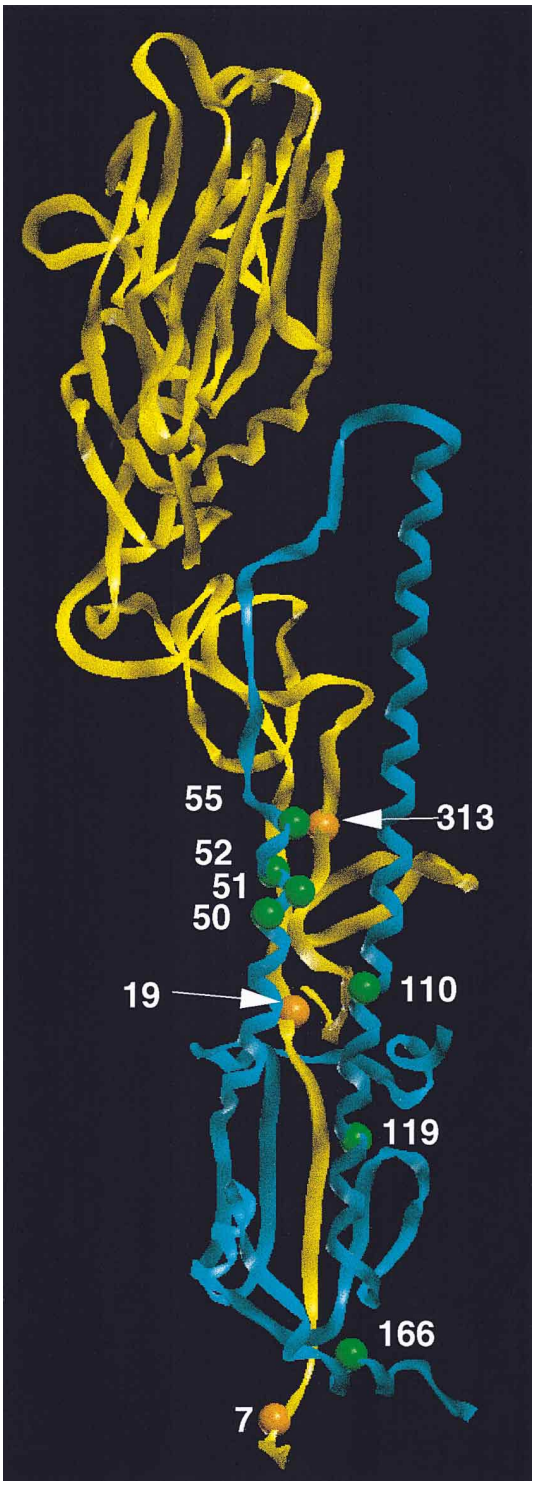


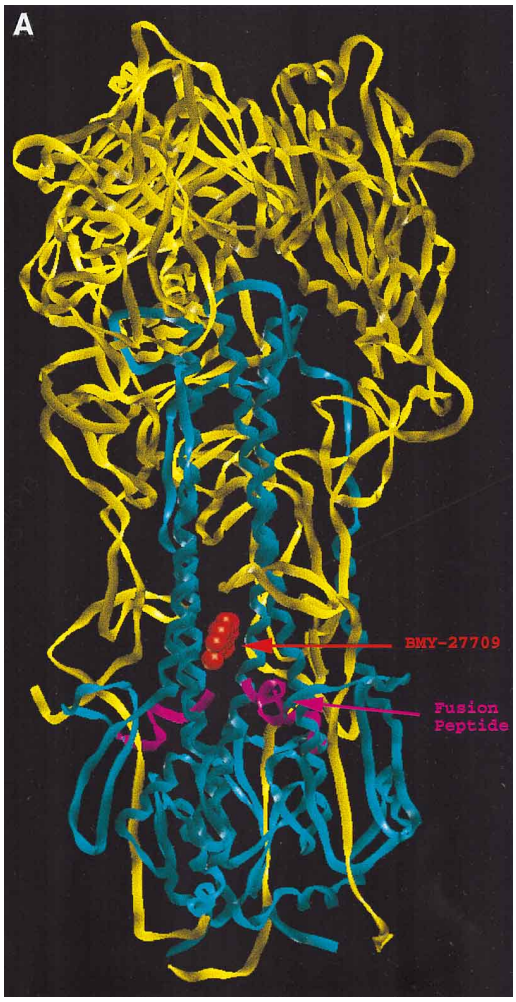
FIG. 5. Locations of amino acid mutations of BMY-27709-resistant viruses in the HA. A ribbon of the HA monomer is shown. Amino acid changes in the resistant A/WSN/33 viruses (H1) were placed on the simulated H1 structure. The amino acid numbering of A/WSN/33 virus is based on the published sequence (18). The amino acid substitutions are indicated by solid circles and numbered according to Table 1. Amino acid substitutions in the HA2 subunit are labeled in green, and changes in the HA1 subunit are labeled in orange.

The results revealed that most resistant viruses are highly resistant to treatment of BMY-27709, with more than a 100-fold increase in their 50% effective concentrations (EC_{50}s) compared to wild-type A/WSN/33 virus (Table 1). However, three resistant viruses still exhibited significant sensitivity to BMY-27709 (Table 1). These include viruses with mutation from tyrosine to histidine at position 7 of the HA1 subunit, asparagine to serine at position 50 of the HA2 subunit, or phenylalanine to leucine at position 110 of the HA2 subunit. Interestingly, viruses with the Phe-to-Leu mutation at position 110 of the HA2 subunit are much less resistant to BMY-27709 than those with the Phe-to-Ser mutation, suggesting that the hydrophobicity of this amino acid is very important for the action of BMY-27709. In addition, all resistant viruses replicate as well as the wild-type virus both in tissue culture and in mice (data not shown). The change in the pH dependence of hemolysis for all of the resistant viruses was also examined. The pH at which A/WSN/33 induced 50% hemolysis is 5.65. All mutations in the HA2 subunit caused an increase or decrease of 0.1 to 0.3 in the pH which induced 50% hemolysis (Table 1). However, the resistance causing mutations in the HA1 subunits did not alter the pH dependence of the HA molecule.

DISCUSSION

The HA-mediated membrane fusion process is a complex and highly cooperative event for which the mechanism has not been completely elucidated (9, 14, 19, 26, 32, 47). It is known that the low-pH-induced structural rearrangement of HA is essential but by itself not sufficient to trigger the fusion process. Mutagenesis studies have revealed that certain changes in the hydrophobic fusion peptide at the amino terminus of HA2 do not affect the conformational change of the proteins in response to low pH but do abolish their membrane fusion (10, 15, 39). This finding suggests that specific amino acids within the fusion peptide are required for the fusion process, although their roles have not yet been determined (4, 12, 29, 33, 37, 39). In addition, little is known about the fusion process after the conformational change of HA. Kinetic studies have indicated that a lag phase precedes fusion, suggesting that a prefusion period is required for the structural transition in response to low pH, which may be necessary for efficient fusion pore formation (1, 2, 8, 14, 19, 26, 36). Biochemical and biophysical studies have suggested that fusion is preceded by the formation, dilation, and merging of fusion pores, in which multiple HA trimers are involved (8, 9, 13, 25, 35, 41, 53). However, a real challenge in understanding the mechanism of HA-mediated membrane fusion is to determine how two separated membranes are brought close enough together to undergo deformation and lipid mixing (7, 14, 32, 36, 47, 52).

In spite of the complexity of HA-mediated membrane fusion, the trigger of the whole process appears to be the structural transition from the native to the fusogenic state of the HA trimer in response to low pH. Membrane fusion is blocked once this trigger is inhibited (3). In this study, we have demonstrated that BMY-27709 inhibits membrane fusion by preventing the native HA trimer from undergoing a low-pH-induced conformational change. The results from an ELISA directly show that the low-pH-induced conformational change of HA, as determined by a low-pH-specific monoclonal antibody (50, 51), was inhibited by BMY-27709. These results were further confirmed by trypsin digestion experiments (Fig. 3), in which drug-sensitive HAs, but not drug-resistant HAs, are protected from trypsin digestion by BMY-27709. We do not know, however, whether BMY-27709 prevents the native HA conformation from undergoing any change at low pH, or



whether initial fusion intermediates of HA do form but are inhibited from undergoing the irreversible transition (1, 2, 21, 28, 36, 40). Further elucidation of the structure stabilized by BMY-27709 at low pH by using monoclonal antibodies specific for various forms of HA (45, 50), X-ray crystallography (42, 49), or other techniques should provide insights into these questions.

One observation is that higher concentrations of BMY-27709 are needed for the inhibition of the low-pH-induced conformational change of HA as measured by ELISA or trypsin susceptibility (Fig. 2 and 3) than inhibition of the HA-mediated membrane fusion as measured by hemolysis and virus growth in tissue culture as measured by MTT staining (Fig. 4B and 4C and reference 24). For instance, the 50% inhibitory concentration (IC_{50}) of BMY-27709 for the inhibition of the HA conformational change of A/WSN/33 virus, as assayed by tryptic digestion (about 80 to 100 μM [Fig. 3]), is about 10 times higher than the EC_{50} (8 to 10 μM) for the inhibition of virus growth (24) or hemolysis (reference 24 and Fig. 4B and C). In the case of influenza A/PR/8 virus, approximately a fourfold difference was observed between the IC_{50} in an ELISA for the HA conformational change (approximately 220 μM [Fig. 2]) and IC_{50} for hemolysis inhibition (50 to 90 μM [reference 24 and unpublished data]). The exact reasons for this phenomenon are not clear. One explanation may be that for membrane fusion, it has been estimated that at least three or four (9) or even more (1, 2, 13, 25, 53) HA trimers are required for fusion pore formation and that even more may be needed for merging of smaller pores to a large one (25, 53). Accordingly, only partial inhibition of the HA molecules present in the fusion pore may be required to block membrane fusion. Both the ELISA and trypsin susceptibility assay examine each HA molecule separately, and thus total inhibition in these assays may require increased compound concentration.

The data described suggest that the inhibitory activity of BMY-27709 is dependent on specific interactions with the HA

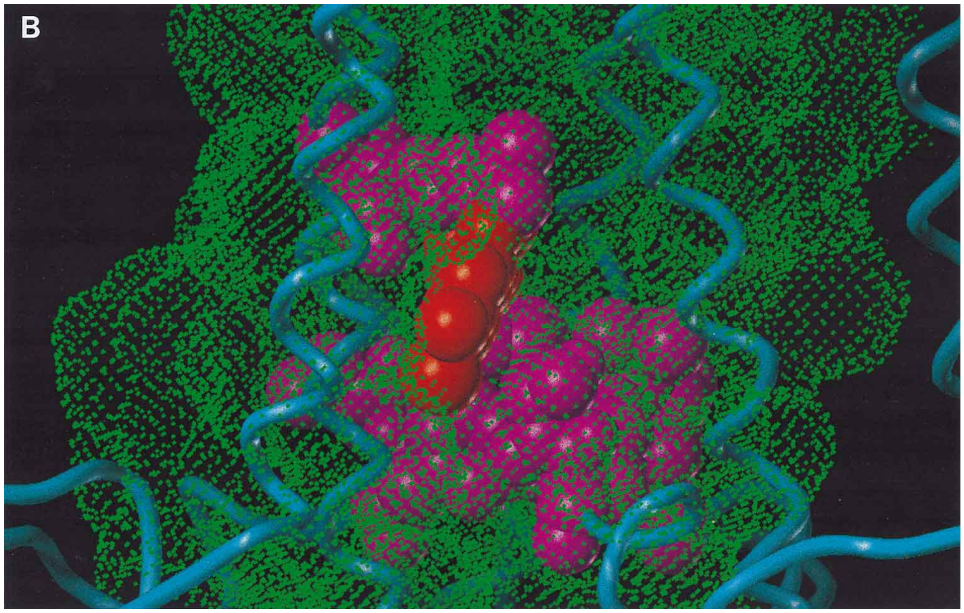


FIG. 6. (A) BMY-27709 docked in the HA trimer of the simulated three-dimensional structure of the H1 HA. The HA1 subunit is in yellow, and the HA2 subunit is in dark blue. BMY-27709 is labeled in red, and the N-terminal fusion peptide of the HA2 subunit is highlighted in magenta. (B) Connolly surface of the proposed binding site. BMY-27709 is shown in red; Glu105 and Arg106 (on top of BMY-27709) and residues 1 to 3 of the fusion peptide (at the bottom of BMY-27709) are highlighted in magenta.

protein. First, it is HA specific, as it is active against H1 and H2 subtypes but not against H3 subtype HAs (24). Genetic analyses of BMY-27709-resistant viruses has revealed that a single amino acid mutation in the HA protein could convert the virus to resistance to BMY-27709 (Table 1). Most of the amino acid substitutions identified in the HAs of the resistant viruses are located in a region near the N terminus of the HA2 subunit, suggesting that a binding pocket for BMY-27709 exists near this fusion peptide. The reversibility of the inhibitory activity of BMY-27709 also suggests that this compound functions through noncovalent interactions with the HA protein (Fig. 1). However, binding to the putative site within the HA protein is not necessarily sufficient for inhibitory activity. Analogs of BMY-27709 with structural changes either in the aromatic ring or on the quanolizidine heterocycle of BMY-27709 (Fig. 4) converted the compound from an agonist to an antagonist. This finding suggests that antagonists BMS-198254 and BMS-195161 can bind to the same pocket as BMY-27709, although neither compound has inhibitory activity. This finding is further supported by the evidence that preincubation of antagonists BMS-195161 and BMS-198254 with the virus did not prevent the binding of BMY-27709, since the virus retains the sensitivity to BMY-27709 treatment (Fig. 4D). Furthermore, both BMS-195161 and BMS-198254 did not significantly ($<$0.1 unit) alter the pH midpoint (at which 50% hemolysis occurred) of the A/WSN/33 virus HA as determined in hemolysis assays (data not shown). However, it may still be possible that these compounds affect the fusion kinetics or transition of HA intermediates at low pH. The data presented, though, are highly suggestive that specific interactions of the aromatic ring moiety as well as the quinolizidine heterocycle of BMY-27709 with the HA protein are required for inhibition of the low-pH conformational change of HA.

Can the above-described data be used to model a mechanism for the action of BMY-27709? Since BMY-27709 is active only against H1 and H2 subtype HAs, whose structures have not yet been determined, the structure of the H1 subtype must be modeled based on the H3 structure (49). The high degree of homology between H1, H2, and H3 subtype HAs (27) allows for a model of the three-dimensional structure of the H1 HA based on the known structure of H3 subtype HA (49). Using the simulated H1 structure, visual examination of the surface of the simulated H1 HA structure reveals a crevice about 18 Å in depth in the region of Phe110. BMY-27709 could be docked to fit compactly into this crevice, as shown in Fig. 6A. The compound binds to the HA protein through hydrophobic and ionic interactions (Fig. 6B). The hydrophobic force came from interactions between the aromatic ring and Phe110 and the quinolizidine heterocycle and the N terminus of the HA2 subunit. The acid surrogate ketophenol moiety of BMY-27709 interacts with Arg106, while the amine in the quinolizidine heterocycle interacts with Glu105 of the HA2 subunit. Mutations which cause resistance map to regions close to this crevice and could either act directly on the binding interactions or cause slight structural rearrangements in the HA. The fact that most of the resistant viruses exhibit an increased pH of fusion suggests that the HAs in these viruses are in a less stable configuration. It should be noted that resistance can be attained through mutations which alter a specific interaction, but not necessarily inhibit binding of BMY-27709, since close analogs can act as antagonists. In that vein, it is not known whether BMY-27709 is able to bind to any or all of the resistant HA proteins.

It is not exactly clear how BMY-27709 prevents the native HA trimer from undergoing the low-pH-induced conformational change. However, inhibition of the transition could occur through a number of mechanisms. Ionic and hydrophobic interactions between BMY-27709 and the HA trimer (Fig. 6B) could immobilize the fusion peptide, thereby preventing the native HA trimer from undergoing the low-pH-induced conformational change. In this scenario, BMY-27709 would act as a "molecular glue" to inhibit movement of the fusion peptide. Alternatively, compound may sit in a region and act as a wedge, directly blocking the movement of either the fusion peptide or other regions of the molecule. Clearly, further structural studies are needed to investigate these possibilities. It is hoped that an understanding of the mechanism of inhibition of BMY-27709 will aid in the elucidation of the events occurring during the low-pH-induced conformational change in the HA protein.

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