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How to overcome resistance of influenza A viruses against adamantane derivatives

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Abstract

We tested two approaches to overcoming resistance of influenza A viruses against adamantane derivatives. First, adamantane derivatives that interfere with the ion channel function of the variant M2 protein of amantadine-resistant viruses may prevent drug resistance, if they are used in mixture with amantadine. Second, amantadine acts on the M2 protein (at low concentrations) and indirectly on the hemagglutinin (at concentrations at least 100 times higher). Identifying and using a drug that reacted with both targets at the same concentration might reduce development of resistance, since, in this case, two mutations, one in each target protein would be necessary at once. Such a double mutation is assumed to be a rare event. We evaluated forty adamantane derivatives and two related compounds to determine whether they interfered with plaque formation by influenza A strains, including A/Singapore/1/57 (H2N2). Variants resistant to drugs that interfered at low concentrations (≈ 1 μg/ml; e.g. amantadine) were cross-resistant with each other, but were sensitive to those agents effective at high concentrations (8 μg/ml; e.g. memantine). The former group of compounds act on the ion channel; the corresponding escape mutants tested had amino acid replacements at positions 27, 30 or 31 of the M2 protein. Hemagglutinin was the indirect target of the latter group of compounds. Variants resistant to these agents lacked amino acid replacements within the ion channel of the M2 protein and the mutants tested had amino acid replacements in the hemagglutinin. Although we failed to identify compounds that interacted with the ion channel of amantadine-resistant variants and inhibited their replication, we were able to construct at least two compounds that interfered with both the ion channel and the hemagglutinin at about the same concentration. After passage in the presence of these compounds, we either failed to obtain any drug-resistant mutants or those obtained had amino acid replacements in the ion channel of the M2 protein and the hemagglutinin.

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1. Introduction

Amantadine is a derivative of adamantane that efficiently inhibits replication of influenza A viruses (Davies et al., 1964). This drug inhibits replication by interfering with the ion channel activity of the matrix (M)-2 protein (Sugrue and Hay, 1991; Pinto et al., 1992; Schroeder et al., 1994) and/or indirectly with the pH-dependent conformational change of the hemagglutinin (HA), which is necessary for fusion of the endosomal and viral membranes (Daniels et al., 1985; Steinhauer et al., 1991) by increasing the endosomal pH. Because influenza viruses (like other RNA viruses) have a relatively error-prone RNA polymerase and no repair system, drug-resistant variants can be obtained easily by passing the virus in the presence of amantadine (Cochran et al., 1965; Appleyard, 1977). The strains obtained by passing the virus in the presence of relatively low concentrations of amantadine (~ 1 μg/ml) contained amino acid replacements at one of four specific sites in the membrane-spanning region of the M2 ion channel (Hay et al., 1985). In contrast, when high concentrations (100 μg/ml) were used, the resistant variants had amino acid replacements at multiple sites within the hemagglutinin molecule (Daniels et al., 1985). With this background experience, we have tested two approaches to overcome drug resistance against adamantane derivatives. Approach 1: Adamantane derivatives that interfere with the ion channel function of the variant M2 protein of amantadine-resistant viruses should prevent drug-resistance, if they are used in mixture with amantadine. Approach 2: A drug that reacts with both targets, with the M2 ion channel and the HA function at the same concentration, might have a reduced probability to develop resistance. In this case, two mutations, one in each target protein would be necessary at once. Such a double mutation is assumed—even with an RNA virus, to be a relatively rare event.

Amantadine is expected to bind to the ion channel region of the M2 protein between amino acid positions 27 and 31 (Duff and Ashley, 1992). Most of the amino acid replacements that lead to amantadine resistance occur at positions 27 (Val), 30 (Ala), and 31 (Ser) (Hay et al., 1985; Bean et al., 1989); a replacement at position 34 (Gly to Asp) was found only once. The amino acids at positions 30 and 31 in the resistant strains are larger than those of the sensitive viruses, while those at position 27 were smaller. This finding led us to hypothesize that adamantane derivatives capable of fitting into the ion channel of the amantadine-resistant strains and interfering with their replication might exist (approach 1). A cocktail of such derivatives might overcome the problem of the appearance of drug-resistant variants and compounds that are very similar in structure probably have similar pharmacokinetics. To identify potentially effective amantadine derivatives, we tested many compounds, some of which are smaller than amantadine and contain 5-member rings in the cage-like configuration of adamantane. Other agents are larger than amantadine and have either 7-member rings arranged in the cage-like structure or have more or less bulky side chains. Some compounds lacked even the cage-like structural motif (Fig. 1). Our data will be discussed together with results obtained already by others, e.g. some amphophilic amines were found to inhibit influenza A virus replication and to interfere with the ion channel activity of the M2 protein, which were not adamantane derivatives like cyclooctylamine (Appleyard and Marber, 1975; Hay et al., 1985; Lin et al., 1997) or the compound BL-1743 (Tu et al., 1996). Several other compounds, active against influenza A viruses, were adamantane derivatives with more complex side groups (Indulen et al., 1979a,b). Of special interest are spiroderivatives (Kolocouris et al., 1994, 1996), which are similar in structure when compared with our compounds 266 and 636 (Fig. 1).

At high concentration, amantadine functions as an amine increasing the pH within endosomes and interfering with the uncoating of the influenza A virus (Kato and Eggers, 1969; Ohkuma and Poole, 1978). Under these conditions the indirect target is HA (Daniels et al., 1985; Steinhauer et al., 1991). At low concentrations the target is the ion channel of the M2 protein (Hay et al., 1985;

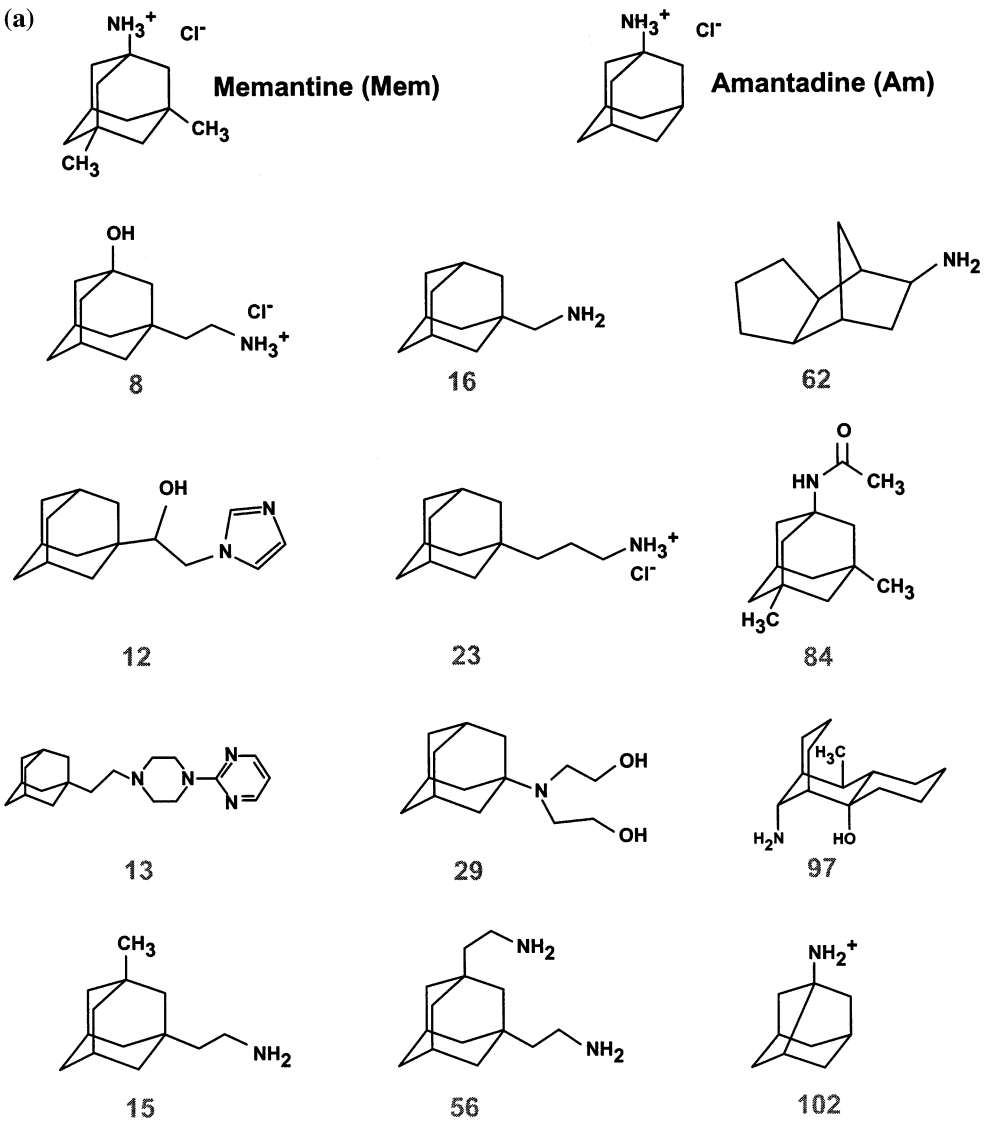
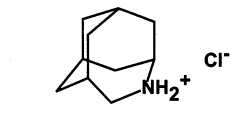


Fig. 1. Structures of the adamantane derivatives and related compounds used in the present study.

Pinto et al., 1992). Therefore, our second goal was to find amantadine derivatives that affected both targets at about the same concentration (approach 2). To this end, we synthesized compounds that contained a secondary or tertiary amino group instead of a primary amino group, thereby increasing the basicity of the derivative and enabling it to affect the hemagglutinin at a lower concentration than does amantadine. At the same time, we added side groups to the ring system. The resulting compounds potentially would affect ion channel activity at increased concentrations because they might fit into the ion channel less tightly.

(b)



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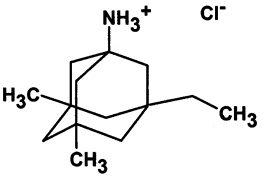

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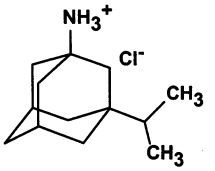

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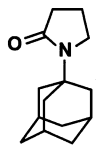

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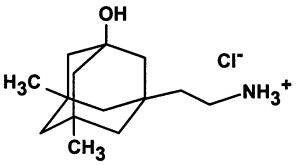

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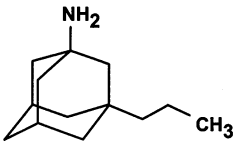

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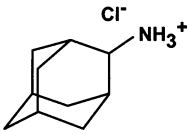

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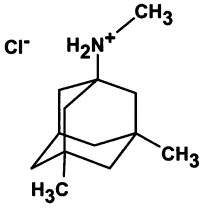

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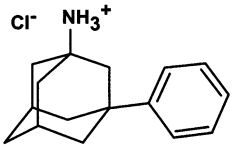

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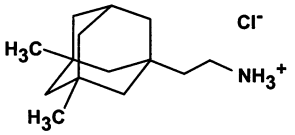

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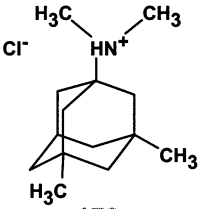

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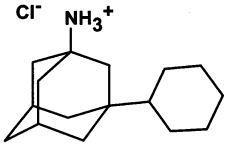

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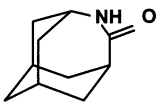

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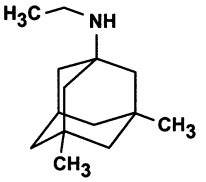

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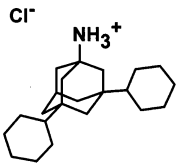

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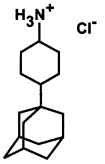

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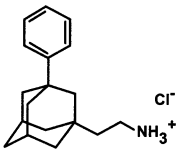

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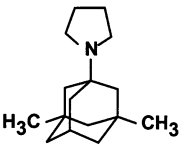

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Fig. 1. (*Continued*)

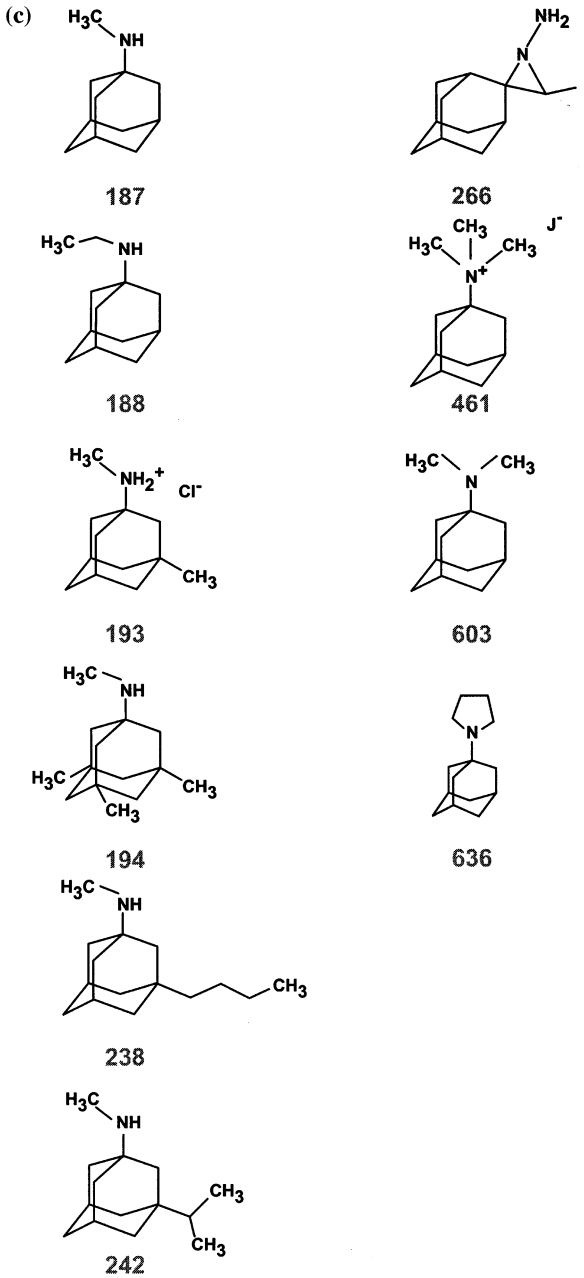

Fig. 1. (*Continued*)

2. Material and methods

2.1. Chemicals

The compounds, as shown in Fig. 1, were synthesized by Merz (Frankfurt, Germany; Bormann et al., 1990). The identity and purity of the compounds were verified (NMR and IR, elemental analysis, GC- and HPLC analysis); data are on file at Merz. All of these compounds were

adamantane derivatives, except No. 62 (5-amino 4,7-methanoperhydroindene) and No. 97 (13-amino-2 hydroxy-8-methyl-tricyclo (7, 3.1.0)-tridecane). Amantadine was obtained from Sigma (St. Louis, MO). Stock solutions were prepared in PBS at a concentration of 1 mg/ml.

2.2. Cells and virus strains

We passed the viruses and performed plaque assays in Madin–Darby canine kidney (MDCK) cells grown in Eagle's minimal essential medium supplemented with 4% bovine serum albumin (BSA).

For single-cycle experiments (multiplicity of infection = $\sim$10 plaque-forming units per cell), the compounds were present 30 min. immediately before, during and for 8 h after infection with A/Singapore/1/57 (H2N2). Further passages were done overnight ($\sim$14 h; $\sim$two replication cycles) in the presence of 1 μg/ml of trypsin. We also tested A/WSN/33 (H1N1), A/Udorn/307/72 (H3N2), A/Swine/1976/31 (H1N1) and B/Ann Arbor/1/86. A/Fowl plague/Rostock/34 (H7N1) was tested in primary chicken embryo cells only in single-cycle experiments. Plaque tests were performed according to Appleyard and Marber (1974) on MDCK cells with 0.5 μg/ml trypsin and the appropriate inhibitor concentration in the 1.8% agar overlay medium.

To isolate drug-resistant variants of A/Singapore/1/57, we picked single plaques and eluted them in 1 ml PBS containing 2% BSA. The medium was aspirated from the cells, then 1 ml PBS and 1, 10 or 100 μl of the plaque solution was added to the well. After 30 min. the cells were washed twice with PBS and overlaid with agar solution containing the compound and 0.5 μg/ml trypsin (Appleyard and Marber, 1974). After 3 days, single plaques were picked again. This procedure was repeated at least twice before we inoculated embryonated chicken eggs with 0.2 ml of the plaque solution to obtain infectious allantoic fluid.

2.3. Polymerase chain reaction amplifications and sequencing

RNA extracted from virus-containing allantoic fluid (Bean et al., 1980) was transcribed into single-stranded cDNA with reverse transcriptase (Life Sciences, St. Petersburg, FL). The cDNA was used as a template in the polymerase chain reaction (PCR) amplifications as described by Katz et al. (1990);(primer sequences available upon request). Samples were amplified in a programmable thermocycler (MJ Research, Watertown, MA), and the DNA products were purified by using the Wizard PCR Prep Kit (Promega, Madison, WI).

We determined the nucleotide sequences of the M and HA genes using the dideoxynucleotide-chain termination method (Sanger et al., 1977). Oligonucleotide primers complementary to the M and HA segments were end-labeled with [^{32}P] ATP (ICN Pharmaceuticals, Costa Mesa, CA), annealed to template DNA and extended by using Taq DNA polymerase in the fmol DNA sequencing system (Promega). The reaction products were separated on a 6% polyacrylamide/7.8 urea gel containing a 1–5X TBE (1 $\times$: 90 mM tris–borate [pH 8.2], 2 mM EDTA) gradient.

2.4. Hemolysis of chicken red blood cells by influenza A viruses

The virus-containing allantoic fluid from 10 embryonated eggs was centrifuged for 15 min at 5000 rpm; the resulting supernatant was layered onto a 25% sucrose cushion, then centrifuged for 90 min at 27 000 rpm. The pellet was resuspended in 1 ml of PBS, sonicated for 2 min and diluted to 10 000 HA units/ml. We prepared fresh solutions of 1% chicken erythrocytes in 0.05 M NaCl buffered with 0.1 M sodium acetate at pHs of 5.0, 5.25, 5.50, etc. We mixed 1 ml of erythrocytes with 40 μl of virus concentrate, incubated the solution on ice for 15 min, then heated at 37°C for 15 min, chilled on ice and centrifuged at 7000 rpm for 7 min. We then determined the optical density at 545 nm of the supernatant (Huang et al., 1981).

Table 1
Inhibition of plaque formation by A/Singapore/1/57 (H2N2)

Minimum dose that completely inhibits plaque formation			Minimum dose that completely inhibits plaque formation		
Compound	(μg/ml)	Target[a]	Compound	(μg/ml)	Target[a]
Am	0.2	M2	154	>10	ni
Mem	8	HA	169	8	HA
8	>10	ni[c]	170	0.5	M2+HA
12	1	M2	171	10	(HA)
13	>10	ni	173	4[b]	ni
15	1	ni	177	1	M2
16	0.2	M2	178	1	ni
23	0.5	M2	180	8	HA
29	4	(M2)[d]	182	>4[b]	ni
56	1	ni	183	>4[b]	ni
62	1	M2	185	>10	ni
84	>10	ni	187	0.5	(M2+HA)
97	4	ni	188	0.5	(M2+HA)
102	0.8	M2	193	0.5	M2+HA
117	1	M2	194	10	(HA)
121	10	ni	238	8	(HA)
125	0.5	M2	242	8	(HA)
138	8	(HA)	266	1	M2
141	>10	ni	461	>10	ni
146	2	ni	603	0.5	(M2)
151	8	(HA)	636	1	(M2+HA)

[a] The target is defined as follows: M2, relatively low concentrations of these drugs interfere with virus replication. Variants resistant to these compounds have a specific amino acid replacement in the ion channel region of the M2 protein (Table 2). These variants are inhibited by memantine (Mem) but not by amantadine (Am); HA, relatively high concentrations of these compounds interfere with virus replication. Resulting variants lack amino acid replacements in the ion channel region of the M2 protein (Table 2). Each contains an amino acid replacement in the hemagglutinin and has an elevated pH optimum for hemolysis of chicken erythrocytes (Table 3). These variants are inhibited by amantadine but not memantine, M2+HA: These compounds inhibit variants resistant to either amantadine or memantine. Escape mutants are difficult to obtain and generally are resistant to amantadine and memantine (double resistance). They have amino acid replacements in the ion channel of the M2 and in the HA protein and they exhibit an elevated pH optimum for the lysis of chicken erythrocytes.
[b] Too toxic to be tested at a higher concentration.
[c] Not further investigated.
[d] Brackets mean that not all of the criteria have been tested.

3. Results

3.1. Inhibition of influenza virus replication by various adamantane derivatives

In single-cycle experiments using MDCK cells, the adamantane derivatives tested (Fig. 1), only negligibly decreased the replication of A/Singapore/1/57, A/WSN/33, A/Udorn/307/72, and A/Swine 1976/31. But in the second cycle, with those compounds which were also active in the plaque-reduction test virus yield of these strains (with the exception of A/WSN/33, which is resistant to amantadine (Hay et al., 1985) dropped to $\sim$10% than that of the control; at this stage in many experiments, the supernatant already contained drug-resistant variants. In contrast, compounds 16, 23, 102, 117, 125 and 170 inhibited replication of A/Fowl plague/ Rostock/34 (H7N1) already in single-cycle experiments using primary chicken embryo cells (data not shown). However, in the following experiments, fowl plague virus was not further studied.

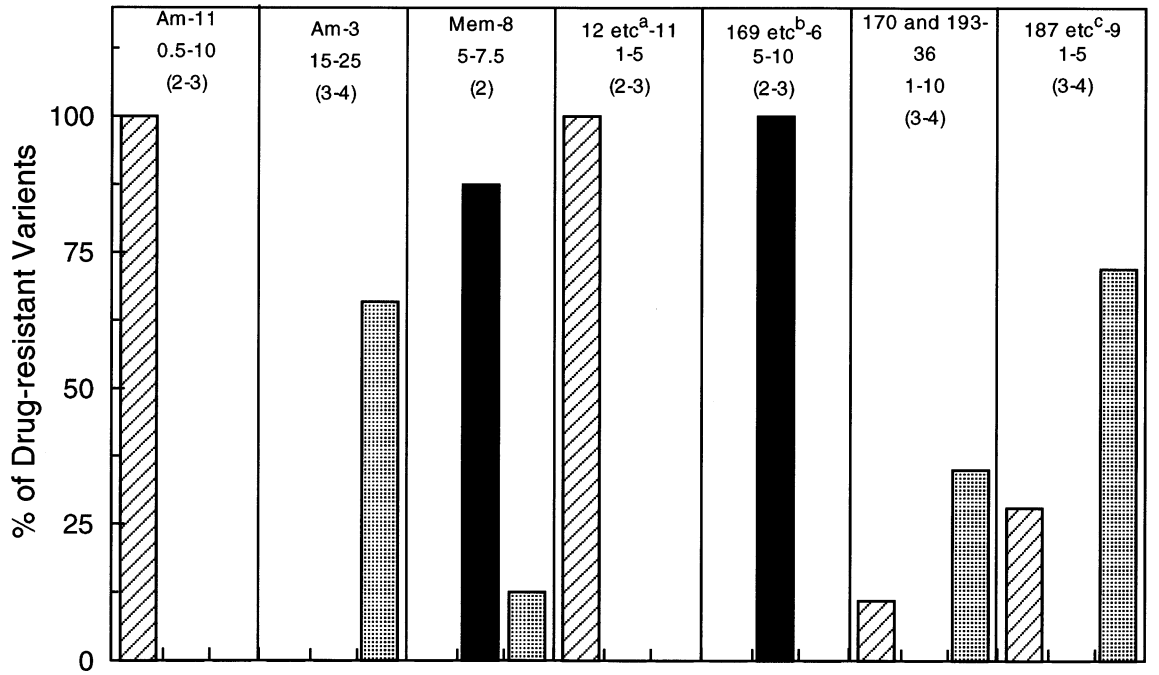

Fig. 2. Drug-resistant variants of A/Singapore/1/57. Besides the total yield after the passage, several individual plaques were tested for resistance to amantadine and memantine. ▨ = resistance to amantadine only; ■ = resistance to memantine only; ▩ = resistance to both, amantadine and memantine. [a] Group comprises compounds 12, 16, 23, 62, 102, 117, 177, 266 and 603; [b] group comprises compounds 169, 180, 238 and 242; [c] group comprises compounds 187, 188 and 636. The figure right of the compounds designates the number of experiments performed. The figures below the compounds' name designate the range of concentration (μg/ml medium) investigated. The figures in brackets designate the number of passages to obtain a drug-resistant virus population.

When we incorporated the adamantane derivatives and related compounds into the overlay medium in plaque tests of A/Singapore/1/57, we obtained the data shown in Table 1. Based on these data, the compounds were placed into one of three groups: (1) Compounds that inhibited plaque formation at relatively low concentrations (~ 1 μg/ml; e.g. amantadine); (2) those requiring relatively high concentrations (~ 8 μg/ml; e.g. memantine) for inhibition of plaque formation; and (3) compounds that failed to interfere with virus replication, even at the highest concentration tested (10 μg/ml). Although compound 62 did not contain the cage-like structure of amantadine, it interfered with plaque formation at relatively low concentrations. Compounds active against A/Singapore/1/57 also inhibited plaque formation by A/Swine/1976/31 and A/Udorn 307/72, but higher concentrations were necessary for the same effect. None of the compounds, including those similar to memantine, prevented plaque formation by A/WSN/33, which is resistant against amantadine. All of the 42 compounds tested also failed to inhibit replication of B/Ann Arbor/1/86 (data not shown).

3.2. Drug-resistant variants of A/Singapore/1/57

Drug-resistant variants of A/Singapore/1/57 were obtained by passing the virus in the presence of various concentrations of the compounds. As shown in Fig. 2, variants resistant to amantadine, but not to memantine, were consistently found latest after the third passage in the presence of 1–10 μg/ml of amantadine and compounds 12, 16, 23, 62, 102, 117, 177, 266 and 603. After passage in the presence of memantine or of compounds 169, 180, 238 and 242, the variants ob-

tained, with only one exception, were resistant to memantine, but not to amantadine or compounds 12, 16, 23, etc. (Fig. 2). Passing A/Singapore/1/57 in the presence of compound 170 or 193, led, in most of the trials, to loss of the virus, latest after the fourth passage. If resistant variants were found, most of them were resistant to amantadine and memantine. Only at relatively low drug concentrations (up to 1.5 μg/ml) in four trials, variants resistant only to amantadine were found. One last group comprising compounds 187, 188 and 636 ranged in their potency to generate drug-resistant variants between 170 and 193 on the one side and amantadine, compounds 12, 16, 23, etc. on the other side. Compounds of this last group are similar in structure to compounds 170 and 193, but the methyl side groups of the ring system are missing.

3.3. Sequencing of the M and HA genes of drug-resistant variants

Low concentrations of amantadine or rimantadine inhibited the ion channel activity of the M2 protein (Pinto et al., 1992; Schroeder et al., 1994), and resulting escape mutants had changes leading to amino acid replacements at one of four sites in the putative ion channel region of the M gene (Hay et al., 1985). For several drug-resistant variants, we sequenced at least nucleotides 626–909, which correspond to about 2/3 of the M2 protein, including the ion channel and part of the M1 protein. The M gene of the A/Singapore/1/57 strain that we used differed from the published sequence (Zebedee and Lamb, 1989) at positions 690 (A→G; His222Arg) and 697 (G→C; Arg224Ser) within the M1 protein. Each of the resistant variants had an amino acid replacement at one of three sites in the ion channel known to be important in resistance to amantadine (positions 27, 30 and 31; Table 2). After being passed in the presence of memantine, an amantadine-resistant strain gave rise to mutants that were also resistant to memantine; however, one of the resulting variants sequenced did not gain a second amino acid replacement in the ion channel region. The mutants that were solely resistant to memantine and were sequenced in the ion channel region lacked an amino acid replacement within the sequenced region of the M2 protein, indicating that compounds like 169, 180 and memantine inhibited influenza virus replication by a different mechanism.

Passage of influenza A viruses in the presence of high concentrations of amantadine led to drug-resistant variants with various amino acid replacements in the HA (Daniels et al., 1985; Steinhauer et al., 1991), leading us to speculate that the memantine-resistant mutants contained similar substitutions. We sequenced most of the HA gene of several of our memantine-resistant variants (obtained after passing A/Singapore/1/57 in the presence of these compounds) and compared the sequence to that of our wild-type virus. The sequenced region spanned from position 15 (Gly) to the carboxyterminus of HA. The sequence of the

Table 2
Amino acid replacements within the ion channel of the M2 protein of drug-resistant variants of A/Singapore/1/57 (H2N2)

Compound	Position and identity of amino acid changes within M2 protein of resistant strains		
	27	30	31
Am			Ser31Asn
Am + Me[a]			Ser31Asn
Me			
12			Ser31Asn
16			Ser31Asn
23		Ala30Tre	
62-1[b]			Ser31Asn
62-2[b]		Ala30Tre	
102			Ser31Asn
117		Ala30Tre	
169			
170-1[b]			Ser31Asn
170-2[b]			Ser31Asn
177	Val27Ala		
180			
193		Ala30Tre	
266		Ala30Tre	

Only those amino acids that differ from those in A/Singapore/1/57 are listed. There were no replacements at position 34. Am, amantadine; Me, memantine.

[a] The Am variant was passed in the presence of memantine. The resulting isolate is resistant to both Am and Me.

[b] Two independently isolated mutants resistant against compound 62 or 170.

Table 3
Drug-resistant variants of A/Singapore/1/57 that required an increased pH for optimal lysis of erythrocytes and that have amino acid replacements in the hemagglutinin

Compound used to obtain the resistant variant	ΔpH	Base substitutions and amino acid replacements in HA
Amantadine[b]	0.05	nd[a]
Memantine[c]	0.15	1212(A→G; Asn390Ser; HA2: position 49); 1238(A→T; Met399Leu; HA2: position 58)
169[c]	0.30	1274(A→T; Ser411Cys; HA2: position 70)
170[d]	0.65	1290(G→A; Arg416Lys; HA2: position 75)
180[c]	0.45	1035(T→C; Leu331Pro; HA1: position 315) 1058(T→C; Ser339Pro; HA1: position 323) 1201(T→C; silent)
193[d]	0.55	1201(T→C; silent); 1240(G→A; Met399 Ile HA2: position 58); 1392(T→C; Phe450Ser; HA2: position 109)

ΔpH: Values are the mean differences between the pH optima for hemoysis by the variants and that of the wild-type virus (pH 5.25). The pH optima varied by ± 0.05 among the three experiments.
[a] Not done.
[b] Resistant only to amantadine.
[c] Resistant only to memantine.
[d] resistant to amantadine and memantine.

wild-type strain we used differed from that published (Schäfer et al., 1993) at positions 539 (A→G; Lys166Glu), 546 (A→G; Glu168Gly, 639 (C→A; Thr199Lys), 877 (T→C; silent) and 1115 (G→A; Val358Ile). The amino acid replacements in the HA of our memantine-resistant mutants are shown in Table 3. Most, but not all of them, are located in the HA2 region. Except for the mutants resistant to compounds 170 and 169, which carry a single amino acid replacement at HA2 position, 75 (Arg→Lys) or 70 (Ser→Cys), respectively, the other mutants have more than one replacement. For the other mutants, it is difficult to decide which of the replacements are responsible or at least contribute to the resistant phenotype.

Many of the resistant mutants obtained by passage at high concentrations of amantadine (Daniels et al., 1985) contained more than one amino acid replacement in the HA. In light of this observation, a change in biologic function cannot be safely correlated with a specific amino acid replacement. Therefore, we decided to characterize most of the memantine-resistant mutants also according to their biologic properties.

3.4. pH-dependent hemolysis by memantine-resistant variants

Amantadine-resistant variants containing amino acid substitutions that reduced the stability of the HA required an elevated pH for optimal fusion and lysis of erythrocytes (Daniels et al., 1985). Therefore, we measured the pH at which our memantine-resistant viruses optimally lysed chicken erythrocytes (Table 3). The pH conditions necessary for maximum hemolysis by variants resistant to 169, 180, 170, 193 and memantine were significantly higher than those for the parent strain; our results are comparable to those obtained by Daniels et al. (1985). In contrast, the Am variant (which is resistant to amantadine but sensitive to memantine) had a pH requirement similar to that of the wild-type virus.

Further, treatment with ammonium chloride (100 μg/ml) increased the pH within the endosomes, thereby inhibiting replication of influenza A viruses. All of the memantine-resistant variants that we have tested thus far, also were unaffected by this treatment (data not shown). Taken to-

gether, these data suggest that memantine, compound 169, compound 180, and related agents interfere with the replication of influenza viruses by affecting the fusion activity of the hemagglutinin only (Table 1).

4. Discussion

Drug-resistant bacteria and viruses cause great concern, and such variants are especially likely among the RNA-containing viruses because of their high mutation rate. Combination of agents with different modes of action may prevent this problem, but drugs with widely different structures might have incompatible pharmacokinetics (e.g. clearance rates, organotropism). Therefore, we propose constructing agents that inhibit variants resistant against a drug that is similar in structure. Such a concept may be testable by using amantadine and its derivatives. Amantadine binds to a specific region of the ion channel of the M2 protein (Duff and Ashley, 1992), and escape mutants contain amino acid replacements that alter the width of the channel (Hay et al., 1985). Therefore, compounds with a more compact or expanded structure might fit into the mutated ion channel and alter its function.

Although a model of the ion channel is available (Sansom and Kerr, 1993), the three-dimensional structure of the M2-protein currently is unknown, so the approach used to develop inhibitors of neuraminidase (von Itzstein et al., 1993) is impossible at present. We empirically compared amantadine derivatives that have a more compact structure (e.g. compound 102) with those having a somewhat expanded structure (e.g. compound 117). We also tested agents with bulky side chains or an open structure (e.g. compound 62; Fig. 1). Many of these compounds interfere with influenza A virus replication at concentrations comparable to those at which amantadine is effective (Table 1). The magnitude of structural differences among these inhibitors is amazing: compound 62, with its open structure, is almost as active as amantadine. There are other compounds lacking the amantadine ring structure and interfering with the M2 ion channel activity, like the spirene-containing compound BL-1743 (Tu et al., 1996) and cyclooctylamine (Appleyard and Marber, 1975; Hay et al., 1985; Lin et al., 1997). In inhibitory compounds, the amino group may stay directly on the adamantane ring like amantadine or compound 125, it might be incorporated into the ring system like compound 117 or cyclooctylamine, or it can be located on side chains as, e.g. in compounds 23, 56 or rimantadine (Indulen et al., 1979b). Reagents like compound 12, in which the nitrogen is part of a ring system quite apart from the adamantane moiety, are active. In contrast, subtle changes (e.g. the introduction of a methyl group) can determine whether the compound interferes with both the HA and ion channel function or with the HA function only (compare compounds 169 and 170). Or, replacement of the methyl group in position three of compound 193 by an isopropyl (242) or n-butyl group (238) abolishes the interference with the ion channel function. On the other side, compound 177, which interferes with the ion channel function, has an isopropyl group in position three, but is missing the methyl group on the amino nitrogen. If an oxygen group is bound to one of the ring carbons (compare compound 8 with 15, 16 or 23; or compound 161 with 117; or compound 121 with 636) the inhibitory activity is lost. Compound 461 with a quaternary amino group is inactive.

Mutants resistant to the various adamantane derivatives, which are cross-resistant to amantadine, have their mutations at the classical positions within the ion channel of the M2 protein (Hay et al., 1985). Even though none of the compounds we tested interfere with the ion channel function of amantadine-resistant variants, such agents may exist. We need to test a greater variety of derivatives which are, as yet, unavailable. Thus, our first approach might still be valid.

Because of its amino group, amantadine at high concentrations elevates the endosomal pH, creating an environment that prevents fusion between the viral and endosomal membranes. This inhibition results because the conformational change of HA that is necessary for fusion cannot occur at the higher pH. Compounds that fail to destroy ion channel function (e.g. memantine) are still

active (albeit at relatively high concentrations) against the second indirect target, HA. The A/WSN/33 strain may be less sensitive to high endosomal pH; inhibition of this strain may require larger drug concentrations than those we tested.

Compounds that interfere with both the ion channel of the M2 protein and HA at similar low concentrations are potentially therapeutically valuable. To escape such a compound, both targets have to be mutated and such an event is likely to be less frequent (approach 2). At least two of the agents we tested, compounds 170 and 193, fulfil this criterion. Compound 170 contains a relatively basic tertiary amino group, whereas compound 193 has a secondary amino group; these groups effectively lower the concentration at which these agents are active against HA. In addition, the side groups present on the adamantane ring system of these compounds may increase the concentration for the ion channel target. On the other hand, these side groups should not be too bulky (see compounds 238 and 242). It remains to be determined how toxic these compounds are in vivo. Tissue culture cells were not affected at these concentrations. The antiparkinsonian agent memantine has been tested in humans. Its toxicity is comparable to amantadine (Danysz et al., 1997). However, compounds with secondary and tertiary amino groups like 170 or 193, respectively, are expected to be more basic. They might accumulate in the acidic compartments of the cell better than amantadine or memantine and thereby, inhibiting non-specifically any receptor-mediated endocytosis (Schlegel et al., 1982) at lower concentrations. Thus, thorough toxicity tests need to be performed with compounds like 170 and 193 before they can be considered for practical use.

The system we used is not ideal because amino acid replacements at any of the multiple sites of HA can circumvent the interference (Daniels et al., 1985). In other words, the likelihood of such escape is high. Thus, although for the escape of compounds 170 or 193, two simultaneous mutations seem to be required, the escape rate is still relatively high. Indeed, most of these escape variants are resistant to both amantadine and memantine, suggesting that mutations in two segments are necessary concurrently for the escape (Fig. 2). Therefore, our rationale and approach are valid, even though the system in which they are demonstrated is imperfect. Although our first approach to find an adamantane derivative which inhibits the replication of an amantadine-resistant influenza A variant was not successful, the idea behind might be of general interest and generally applicable. The second approach, to find a compound which interferes with two targets at about the same concentration, like compounds 170 and 193, might be of interest for influenza A viruses to minimize the appearance of drug-resistant variants. However, there are certainly not many systems to which this approach could be applied.

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